THE RELATION TO THE BLOOD OF THE VIRUS OF EPIDEMIC POLIOMYELITIS.*

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Early in the history of experimental poliomyelitis Flexner and Lewis[1] succeeded in one instance in producing infection in a Macacus monkey through the intravenous injection of twenty-five cubic centimeters of the defibrinated blood obtained from a recently paralyzed monkey. When a smaller quantity (ten cubic centimeters) of the blood was injected into the circulation, or still smaller quantities were introduced intracerebrally, paralysis did not follow. Leiner and von Wiesner[2] at first reported only negative results from the injection of blood from both human and experimental cases of poliomyelitis; but later[3] they observed in one instance paralysis following the injection, partly intracerebral and partly intravenous, of defibrinated blood taken from a monkey on the third day of paralysis. Römer[4] and Landsteiner and Levaditi[5] also record only failure both with the blood of human and of experimental cases of the disease.

The subject of the relation to the blood of the virus of poliomyelitis is of more than theoretical interest, as it may have a bearing on the manner of transmission of the disease. On that account we have carried out a larger series of experiments in order to determine, as far as possible with the methods at present available, what this relation is.

The first series of inoculations was performed with blood taken

* Received for publication, December 20, 1913.

1 Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

2 Leiner, K., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698.

3 Zappert, J., von Wiesner, R., and Leiner, K., Studien über die Heine-Medinsche Krankheit, Leipzig and Vienna, 1911, 171.

4 Römer, P. H., *München. med. Wchnschr.*, 1910, lvii, 229.

5 Landsteiner, K., and Levaditi, C., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 833.

from human cases admitted to the Hospital of The Rockefeller Institute.

SURVIVING HUMAN CASES.

Case 1.—N. B. Age, 21 years. Onset Oct. 1, 1910. Condition: weakness and atrophy of muscles of right shoulder girdle and arm, atrophy of muscles of right thigh. Blood and spinal fluid normal. Blood collected 24/24.[6]

Oct. 25. 2 c.c. of defibrinated blood injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus*. No symptoms of poliomyelitis developed.

Case 2.—J. H. Age, 12½ years. Onset Oct. 16, 1910. Condition: partial paralysis of legs; temperature 101.6° F. Oct. 27. Increased globulin in spinal fluid. Blood collected 13/9.

Oct. 27. Immediately after collection 3 c.c. of the whole blood were injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus*. No effects followed.

Case 3.—J. A. Age, 20 years. Onset July 14, 1911. Condition: complete paralysis of right and partial paralysis of left leg. Increased cells and globulin in spinal fluid. July 22. Blood collected and defibrinated 9/7.

July 22. 4 c.c. of the blood were injected intracerebrally and 10 c.c. intraperitoneally into a *Macacus rhesus*. Monkey remained well.

Case 4.—M. B. Age, 5 years. Onset Oct. 3, 1912. Condition: temperature 104.8° F.; right leg paralyzed; left leg weak; neck and back stiff; facial muscles on left side weak. Increased cells in spinal fluid. Oct. 8. Right arm paralyzed. Blood collected 6/3.

Oct. 8. The whole blood was immediately injected as follows: 2 c.c. intracerebrally, and 30 c.c. intraperitoneally into a *Macacus rhesus*. No ill effects followed.

Case 5.—A. J. Age, 10 years. Onset Oct. 13, 1912. Condition: tenderness in limbs and back; left arm weak; right shoulder and extensors of right arm paralyzed; intercostal muscles on both sides paralyzed. Oct. 23. Blood collected 11/7.

Oct. 23. Injected 30 c.c. of whole blood into the femoral vein of a *Macacus rhesus*. Monkey remained well.

Case 6.—H. O'C. Age, 3 years. Onset Nov. 1, 1912. Condition: temperature 101.8° F.; right leg paralyzed; intercostal muscles on left side weak; muscles of anterior abdominal wall and anal sphincters weak. Increased cells in spinal fluid. Nov. 5. Blood collected 5/4.

Nov. 5. 2 c.c. of whole blood were injected into the left sciatic nerve of a *Macacus rhesus*. No symptoms of poliomyelitis developed.

FATAL HUMAN CASES.

Case 7.—G. G. Age, 9½ years. Onset Aug. 23, 1911. Aug. 30. Condition: temperature 102.2° F.; legs completely, arms partially paralyzed; intercostal muscles completely, diaphragm partially paralyzed. Sept. 1. Death. Blood collected aseptically from the heart post mortem.

[6] In each case the numerator of the fraction denotes the day of the disease and the denominator the day of the paralysis on which the blood was collected from the arm vein for injection.

Sept. 2. Injected 3 c.c. of the defibrinated blood intracerebrally and 12 c.c. intraperitoneally. Monkey remained well.

Case 8.—J. L. Age, 5½ years. Onset July 12, 1912. July 20. Condition: temperature 105° F.; legs and muscles of shoulders paralyzed; facial muscles of right side weak; intercostal muscles paralyzed. Death same day. Blood collected aseptically from heart post mortem.

July 21. 30 c.c. of citrated blood were injected intraperitoneally into a *Macacus rhesus* monkey, and 0.75 c.c. intracerebrally and 30 c.c. intraperitoneally into another *Macacus rhesus*. Neither monkey developed symptoms of poliomyelitis.

Case 9.—S. N. Age, 6 years. Onset Sept. 28, 1912. Sept. 30. Condition: patient cyanotic and gasping for breath; intercostal muscles paralyzed and diaphragm weak. Increased globulin and cells in spinal fluid. Died same day. Blood collected aseptically from heart post mortem.

Case 10.—G. G. Age, 20 months. Onset gradual and indefinite. Sept. 30, 1912. Intercostals paralyzed; shoulder muscles and facial muscles on right side weak. Oct. 2. Death. Blood collected from heart post mortem.

Case 11.—M. K. Age, 2 years. Onset Sept. 28, 1912. Oct. 1. Temperature 102.4° F.; legs completely paralyzed; intercostals weak; pulse weak and irregular. Increased cells and globulin in spinal fluid. Oct. 4. Consolidation of both lungs. Temperature 104.5° F. Death on same day. Blood collected from heart post mortem.

Oct. 11. Injected 2.5 c.c. of the mixed defibrinated blood of these three fatal cases intracerebrally, and 7 c.c. intraperitoneally into a *Macacus rhesus* which showed excitability on Oct. 15. No paralysis occurred; and later a test inoculation with M A virus caused typical paralysis. Hence it was concluded that this monkey had not developed an abortive attack of experimental poliomyelitis from the injection of the blood.

Case 12.—J. C. Age, 9 years. Onset Oct. 5, 1912. Oct. 8. Condition: temperature 102.6° F.; muscles of right leg and both shoulder girdles paralyzed; intercostal muscles weak. Increased cells and globulin in spinal fluid. Oct. 9. Death. Blood collected from heart post mortem.

Oct. 9. 2 c.c. of the whole blood injected intracerebrally and 35 c.c. intraperitoneally into a *Macacus rhesus*. No effects followed.

These tests confirm those previously made by others with the blood from human cases of poliomyelitis. They do not, however, suffice to exclude altogether the possibility of the virus being present in the blood, since it is known that the original human strains are not as infectious for monkeys as the adapted strains. Another complicating factor may sometimes play a part in preventing infection from the blood. After the first week of acute illness immune bodies which have a neutralizing effect on the virus appear in the blood, and it is not improbable that by acting on the virus, originally of low infective power for monkeys, they may further diminish the chances of producing paralysis. In view of these considerations

we have turned to the experimental cases of poliomyelitis in order to find a solution of the question.

EXPERIMENTAL.

In several instances in the course of our experimental study of poliomyelitis we have made inoculations of the blood of monkeys in the acute stage of paralysis into the brain or peritoneum of normal monkeys in order to ascertain whether the virus is thus demonstrable in the circulating blood. They all resulted negatively.

The experiments were now modified as follows: heavy emulsions of the spinal cord taken from a recently paralyzed animal were injected (a) intracerebrally and (b) both intracerebrally and intraspinously into monkeys from which blood was taken at intervals for inoculation intracerebrally into other monkeys. It was reasoned that the virus would be carried from the subarachnoid spaces into the veins of the membranes of the spinal cord and thence into the general blood. The protocols follow.

Experiment A.—Mar. 24, 1913. 3 c.c. of a suspension of glycerinated M A spinal cord were injected intracerebrally into a *Macacus rhesus*. Blood was taken one, six, twenty-four, and forty-eight hours after inoculation. Mar. 31. Paralysis of arms and back. Apr. 2. Etherized. With the blood taken at the intervals given other monkeys received intracerebral injections of 1 c.c. each. None developed symptoms of poliomyelitis.

Experiment B.—May 16, 1913. A *Macacus rhesus* was injected intracerebrally with 2 c.c., and intraspinally with 5 c.c. of emulsion of the spinal cord (M A) taken from a paralyzed monkey. Blood was taken one, six, and twenty-four hours after inoculation for reinoculation into other monkeys. May 24. Paralysis and prostration; etherized. With the blood withdrawn at intervals, from 2 to 3 c.c. were injected intracerebrally into *Macacus rhesus* monkeys, none of which showed symptoms of poliomyelitis.

Hence under conditions favorable for the passage of the virus into the blood none could be detected by the experiments performed. However, as will appear later, the negative results obtained might have been due in part to the relatively weak virulence of the strain of virus employed since the M A virus had at this period lost a large part of its activity.[7] When a highly active virus is employed for intracerebral inoculation it has happened exceptionally that the

[7] Flexner, S., Clark, P. F., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 195.

blood is infective when taken early in the paralysis or at the onset of symptoms,[8] as is shown in the following protocol.

Experiment C.—A *Macacus rhesus* had been inoculated intracerebrally with K virus on Nov. 17, 1913, and on Nov. 24 had begun to show definite early symptoms of infection. Blood was immediately withdrawn and defibrinated, and 4 c.c. were injected intracerebrally into another rhesus monkey which on Nov. 29 was somewhat excitable. On Nov. 30 the latter showed paralysis of the left leg. On Dec. 1 the arms and back were weak. On Dec. 2 animal died. Typical lesions of poliomyelitis were present.

We next resorted to the active K virus for the double inoculation.

Experiment D.—Jan. 5, 1914. A needle was introduced into the lumbar cistern of the spinal cord of a *Macacus rhesus,* and a few drops of clear cerebrospinal fluid were permitted to escape. While in position 2 c.c. of an emulsion of K virus were injected intracerebrally. The injection was followed at first by the escape of clear fluid, and in two and a half minutes turbid blood-tinted fluid escaped from the lumbar puncture needle. On completion of the intracerebral inoculation 2 c.c. of the emulsion were injected intraspinously. Five hours and seventy-two hours later blood was taken from the median basilic vein and defibrinated for intracerebral inoculation. Jan. 8. Excitable; left arm and back weak; nystagmus. Jan. 10. Paralysis of arms. Jan. 12. Death. Lesions of poliomyelitis present.

Two Macacus monkeys were injected intracerebrally, one with the defibrinated blood taken five hours, and the other three days (at onset of paralysis) after the double inoculation. Both remained well.

This experiment indicates that the detection by inoculation of even a highly pathogenic virus in the blood following combined intracerebral and intraspinous injection is difficult and uncertain. That the virus may sometimes appear in the blood after intraspinous inoculation is shown by the detection of the globoid microörganisms in the film preparations in one such instance.[9]

The problem was now approached from another side. The virus was injected into the veins and the blood tested subsequently in order to ascertain whether it remains and multiplies there or is filtered out and removed. While doubt may exist as to whether the virus enters the blood in quantity and remains there in the case of intracerebral inoculation there can be no doubt of the entrance by the intravenous mode of inoculation, in which the quantity introduced may also be varied at will. Hence a series of experiments

[8] Zappert, J., Leiner, K., and von Wiesner, R., *loc. cit.*

[9] Amoss, H. L., *Jour. Exper. Med.,* 1914, xix, 212.

was made by injecting the virus intravenously and withdrawing blood at intervals from a distant vein and injecting it intracerebrally into normal animals.

Experiment E. Macacus rhesus 1.—Oct. 30, 1913, 3 P. M. 180 c.c. of a filtrate of K virus were injected into the right external saphenous vein. 4 P. M. Sufficient blood was withdrawn from the left external saphenous vein to yield 4 c.c. after defibrination. Oct. 31, 3 P. M. The same quantity was withdrawn from the left leg, and the defibrinated blood specimens were inoculated into two *Macacus rhesus* monkeys, 2 and 3. Nov. 9. Very weak; excitable. Died during night. Lesions of poliomyelitis present in spinal cord. Control died in six days. Typical lesions.

Monkey 2.—Oct. 30. One hour blood specimen inoculated intracerebrally. Nov. 9. Excitable and weak. Nov. 10. Left facial paralysis; ataxic. Nov. 12. Prostrate. Etherized. Lesions of poliomyelitis were present.

Monkey 3.—Oct. 31. 24 hour specimen inoculated intracerebrally. Nov. 10. Excitable; back and arms paralyzed. Nov. 11. Prostrate. Etherized. Lesions of poliomyelitis were present.

Experiment F. Macacus rhesus 1.—Nov. 14, 1913, 10.50 A. M. 100 c.c. of a filtrate of K virus were injected into a vein of the right leg. 12 M. Sufficient blood was withdrawn from a vein in the left leg to yield 4 c.c. after defibrination. Nov. 15, 11 A. M. Same quantity of blood withdrawn. Nov. 16, 11 A. M. Same quantity withdrawn. Nov. 17, 11 A. M. Same quantity withdrawn. Nov. 24. Excitable; left leg paralyzed; left facial paralysis. Sufficient blood withdrawn to yield 4 c.c. when defibrinated. Nov. 25. Legs, arms, and back weak or paralyzed. Dec. 1. Died. Lesions of poliomyelitis were present.

Monkey 2.—Nov. 14. One hour blood specimen was injected intracerebrally into a *Macacus rhesus*. Nov. 18. Excitable; left facial paralysis. Nov. 19. Extremities paralyzed. Nov. 20. Prostrate. Etherized. Lesions of poliomyelitis.

Monkey 3.—Nov. 15. 24 hour specimen inoculated intracerebrally. No symptoms developed.

Monkey 4.—Nov. 16. 48 hour specimen inoculated intracerebrally. Nov. 22. Excitable. Arms paralyzed. Nov. 24. Prostrate. Died. Lesions of poliomyelitis.

Monkey 5.—Nov. 17. 72 hour specimen inoculated intracerebrally. Nov. 24. Excitable. Arms and legs paralyzed. Blood was withdrawn for reinoculation, and employed in experiment C (page 227). Etherized. Lesions of poliomyelitis were present.

Monkey 6.—Nov. 24. 4 c.c. of defibrinated blood, taken 10 days after the intravenous injection of the filtrate when paralysis appeared, was inoculated intracerebrally into monkey 6. No symptoms appeared.

These experiments show clearly that when large and overwhelming quantities of an active filtrate are injected into the circulation the virus persists in the blood for seventy-two hours at least. Finally, it appears to be removed, as ten days after its injection, at the period of the onset of paralysis, it may not be detectable by

the inoculation test. The failure of the monkey inoculated with the twenty-four hour specimen in experiment F to respond may have been due to resistance in the animal rather than to absence of the virus; however, this animal when subsequently tested with an emulsion became typically paralyzed. That the ten day specimen of blood was no longer infectious is not remarkable in view of the many negative results from inoculations of blood at the beginning of the paralysis; but the successful result (experiment C, page 227) in which the blood was infectious at the onset of the paralysis seven days after an intracerebral injection shows that the active virus may pass into the blood from the nervous tissues in which it has multiplied and may survive there for a time.

The infectivity of the blood is affected by the amount of virus injected. Experiments were made also with intravenous injections of ten cubic centimeters of filtrate.

Experiment G.—Dec. 15, 1913. 10 c.c. of filtrate of active K virus were injected into the right external saphenous vein of a *Macacus rhesus*. Dec. 16 (24 hours later) and Dec. 17 (48 hours later) 5 c.c. of blood withdrawn from the left external saphenous vein were defibrinated and each lot was injected intracerebrally into a *Macacus rhesus*. None of the three monkeys of this series developed symptoms.

Experiment H. Macacus rhesus 1.—Jan. 5, 1914. 10 c.c. of filtrate of active K virus were injected intravenously. Six and twenty-four hours later 5 c.c. of blood were withdrawn, defibrinated, and injected separately into *Macacus rhesus* monkeys 2 and 3. The animal receiving the intravenous injection showed indefinite symptoms of excitability but developed no paralysis; it gradually became thinner and weaker and died on the twenty-first day after the injection. No apparent cause of death was found in the viscera. No lesions of poliomyelitis existed.

Monkey 2.—Jan 5. Received the 5 hour specimen of blood intracerebrally. Jan. 14. Excitable; arms paralyzed. Jan. 15. Legs paralyzed; prostrate. Jan. 17. Dead. Lesions of poliomyelitis present.

Monkey 3.—Jan. 6. Received the 24 hour specimen of blood intracerebrally. Jan. 14. Excitable; left arm weak. Jan. 15. Arms, legs, and back weak or paralyzed. Jan. 18. Prostrate. Jan. 24. Prostrate, moribund. Death hastened by ether. Lesions of poliomyelitis were present.

Experiments G and H bring out clearly the uncertainty of the intravenous mode of inoculation first in causing paralysis and second in maintaining the infectivity of the blood. It would appear that only when the blood is overwhelmed by the virus is it certainly infectious over a period of three days or less. Moreover, the failure

of an intravenous dose of ten cubic centimeters of filtrate of K virus to cause paralysis when 0.2 of a cubic centimeter or less is a certainly effective dose by intracerebral injection, not only emphasizes the relative susceptibilities of monkeys to the two modes of inoculation but indicates also the possession of mechanisms by the body capable of excluding the virus within the blood from the nervous tissues. Whether it is the choroid plexus that is responsible or some other structure can only be surmised. It is conceivable that access of the virus to the central nervous system is secured only by way of the cerebrospinal fluid, in which case the virus within the blood must first penetrate the barrier of the choroid plexus which possibly takes place only when overwhelming doses are injected intravenously. When the virus is successfully inoculated subcutaneously or intraperitoneally it is always possible that the penetration to the central nervous organs is by way of the nerves. Hence the results may bear on the mode of infection of poliomyelitis in man.[10] In this connection it may be stated that the incubation period of the disease is longer with intravenous than with intracerebral inoculation. With the latter the average in a series of ten animals was 6.6 days and with the former in a series of six animals it was 10 days.

That the degree of virulence of the strain has a share in the effects is clearly illustrated by an experiment carried out with M A virus at a time when its infectious power had diminished.

Experiment I.—Oct. 3, 1913. 100 c.c. of filtrate of M A virus were infused into the leg vein of a *Macacus rhesus*. One hour and twenty-four hours later blood was withdrawn from the opposite leg and defibrinated. 3 c.c. were inoculated intracerebrally into each of two *Macacus rhesus* monkeys. None of the monkeys inoculated showed symptoms.

This experiment may have a bearing upon the unsuccessful inoculations with blood from human cases. There is little doubt of the presence of the virus in the one hour and the twenty-four hour samples of blood; and yet the quality of the virus was such that it failed to cause infection even when employed for intracerebral inoculation.

[10] Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

EXPERIMENTS WITH STOMOXYS CALCITRANS.

To the data on the infectivity of the blood given in the preceding pages there may be added briefly the results of a few experiments carried out with *Stomoxys calcitrans*. Rosenau and Brues[11] reported several instances of successful transmission of experimental poliomyelitis by means of the biting stable fly, a fact that was at first quickly confirmed by Anderson and Frost.[12] Through the courtesy of Dr. Rosenau one of us (Clark) was enabled to study his method of experimentation so that in the tests made by us Rosenau's method could be followed. Highly active M A virus was used in the experiments.

Experiment 1.—Oct. 1, 1912. 2 c.c. of a suspension of the spinal cord were inoculated intracerebrally into a *Macacus rhesus*. Oct. 4. Excitable. Oct. 6. Partial paralysis. Oct. 7. Prostrate. Oct. 9. Death.

On Oct. 4 and each day thereafter until Oct. 9 about 200 *Stomoxys calcitrans* were permitted to feed on the inoculated monkeys for two or three hours daily. The stable flies were caught in stables in New York City, and the losses from death were made up by adding fresh flies from time to time. The total number of flies employed in the experiment was about 400.

Oct. 7. A *Macacus rhesus* was inoculated intracerebrally with M A virus. Paralysis occurred on Oct. 16, prostration on the 17th, and death on the 18th. The same flies that fed on the preceding monkey were allowed to feed on this animal on Oct. 12 and again on Oct. 14, 15, 16, and 17. These flies were, therefore, permitted to feed ten times upon infected monkeys within fourteen days, five of the feedings taking place before, and five after the onset of paralysis.

From Oct. 6 to 18, except on three days, the 12th, 15th, and 16th, when fresh flies were introduced into the cage and fed first on infected animals, all these flies were given access, for two to three hours at a time, to four healthy monkeys (two *Macacus rhesus* and two *Macacus cynomolgus*). The healthy monkeys were therefore exposed to the flies ten times, covering a period of thirteen days. None of the monkeys developed symptoms of poliomyelitis.

At the conclusion of the feedings the dead flies were collected, ground up, and converted into a Berkefeld filtrate which was inoculated intracerebrally into a *Macacus rhesus* with negative result.

Experiment 2.—Six *Macacus rhesus* monkeys were inoculated intracerebrally between Oct. 18 and Nov. 9, 1912, with M A virus, and a *Macacus cynomolgus* with tonsils from a fatal human case of poliomyelitis. The rhesus monkeys became paralyzed and succumbed; the cynomolgus developed a partial paralysis and was etherized.

Stomoxys were allowed to feed on at least one of the monkeys of this series twenty-one times between Oct. 22 to Nov. 17, or a period of twenty-seven days.

[11] Rosenau, M. J., and Brues, C. T., Fifteenth International Congress of Hygiene and Demography, Washington, 1912.

[12] Anderson, J. F., and Frost, W. H., *Public Health Reports*, 1912, xxvii, 1733.

Monkeys in the preparalytic and paralytic stages were exposed to the flies. Since the death losses were large, about 1,400 flies in all were used in the experiment.

Beginning on Oct. 25 two healthy animals, one *Macacus rhesus* and one *Macacus cynomolgus,* were exposed to the bites of this lot of flies, for two to three hours at each exposure, on twenty-one days. The rhesus monkey appeared somewhat excitable on Nov. 4, and the excitability persisted until Nov. 11, when an examination of the cerebrospinal fluid showed it to be normal. No paralysis developed. The cynomolgus became somewhat excitable and developed weakness of one arm on Nov. 1. The condition did not progress, and on Nov. 4 the animal was etherized. Reinoculation of the spinal cord gave a negative result, and microscopical examination of the spinal cord and intervertebral ganglia showed them to be normal. Possibly the arm may have been hurt by the handling of the animal. This cynomolgus was replaced by another which was exposed to the flies from Nov. 4 to 17. It remained well.

At the conclusion of the experiment a filtrate was prepared from the dead flies, which was without effect when inoculated intracerebrally into a *Macacus rhesus.*

Experiment 3.—About 100 Stomoxys were allowed to feed on a *Macacus rhesus* during the two days following injection of a suspension of M A virus (spinal cord), and 150 Stomoxys were permitted to feed on a paralyzed and moribund monkey. The two lots were ground together and the filtrate from this emulsion was inoculated intracerebrally and intraperitoneally into a *Macacus rhesus.* The animal remained well.

The experiments conducted with Stomoxys gave negative results. In view of the tests on the infectivity of the blood given in the first part of this paper they are quite comprehensible, since it is only under exceptional circumstances that in intracerebrally inoculated monkeys the virus can be demonstrated in the blood by inoculation tests. The negative character of the tests with Berkefeld filtrates prepared from the bodies of the dead flies derives some significance from the single and exceptional successful inoculation with a similar filtrate prepared from *Cimex lectularius* (the bedbug), as reported by Howard and Clark.[13] Finally, it should be stated that a second series of experiments with the stable fly conducted by Anderson and Frost[14] was wholly negative, as were the comprehensive and critical experiments carried out by Sawyer and Herms.[15]

[13] Howard, C. W., and Clark, P. F., *Jour. Exper. Med.,* 1912, xvi, 850.
[14] Anderson, J. F., and Frost, W. H., *Public Health Reports,* 1913, xxviii, 833.
[15] Sawyer, W. A., and Herms, W. B., *Jour. Am. Med. Assn.,* 1913, lxi, 461.

SUMMARY.

Specimens of human blood taken during the paralytic stage of poliomyelitis and post mortem have proved not to be capable of infecting Macacus monkeys.

Specimens of monkey blood taken at various stages of experimental poliomyelitis have not proved as a rule to be capable of infecting monkeys. In a single instance, among ten tests, infection was secured with a specimen of blood removed at the beginning of the paralysis on the seventh day following an intracerebral inoculation.

When suspensions of the spinal cord from a paralyzed monkey have been injected into the brain or simultaneously into the brain and spinal canal, the blood removed from one to forty-eight hours later failed to cause paralysis after intracerebral injection.

When large volumes of active filtrate are injected into the circulation the blood remains infective for seventy-two hours at least, but may be no longer infective after ten days when the paralytic symptoms first appear. When, however, the filtrate is injected in smaller amount or when a filtrate of a less active virus is employed in large quantity, the blood either fails to convey infection or conveys it irregularly.

It is only when overwhelming quantities of an active virus are injected into the blood that paralysis results. The injection of moderate doses is not followed by paralysis, although the virus may still be detected in a blood sample twenty-four hours after the injection. The existence of a mechanism capable of excluding the virus within the blood from the central nervous organs is therefore inferred.

Infection is accomplished far less readily through the circulation than by means of the more direct lymphatic and nervous channels of communication with the central nervous system.

Several series of feeding experiments conducted with the biting stable fly (*Stomoxys calcitrans*) resulted negatively.

THE RELATION BETWEEN THE FAT CONTENT OF THE BILE AND FATTY CHANGES IN THE LIVER.*

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Although numerous analyses of bile are reported in the literature, relatively few deal with the chemistry of the bile in the pathological state, and little systematic investigation seems to have been made of the bile in diseased conditions of the liver. A few observations are recorded, most of them old, which seem to show that, in a general way, acute inflammatory conditions of the liver lead to an increased secretion of the bile constituents, although the amount of water eliminated may be small, while atrophy and chronic degenerations result in a much lowered secretion.

Concerning the composition of the bile secreted by fatty livers, we have found only a few observations. von Gorup-Besanez[1] saw fat droplets and palmitin crystals in the bile in cases of typhoid fever and tuberculosis. Ritter,[2] in two publications on the composition of colorless bile, found a normal, unvarying fat content of the gall bladder bile in what were diagnosed macroscopically as "fattily degenerated" livers, three in man and one in a dog. However, as the livers were not reported as being excessively fatty, that is, fattily infiltrated, and as no analysis for fat was made, the diagnosis being macroscopic only, and as the determination of fat and cholesterol only without consideration of the fatty acid content of the soaps of the bile, seems to have been made, as far as we can tell from the published figures, it is doubtful if the results are of much value as an indication of the general condition of the bile in fatty changes in the liver. Accordingly it seems that Hoppe-

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[1] von Gorup-Besanez, E. F., Lehrbuch der Chemie, 3d edition, Braunschweig, 1874, iii, 525.

[2] Ritter, E., *Jour. de l'anat. et de la physiol.*, 1872, viii, 181; *Compt. rend. Acad. d. sc.*, 1872, lxxiv, 813.

Seyler's[3] quotation of Ritter's results as settling the question of the composition of bile secreted by fatty livers is unwarranted. Hoppe-Seyler states in the same passage that the bile of geese may remain normal in fat composition in high degrees of fatty infiltration of the liver. On the other hand, more recently, Gilbert and Jomier[4] have published the results of investigations in which an excretion of considerable amounts of fat in the bile was observed histologically in experimental fatty infiltration of the liver in rabbits, following the ingestion of meals rich in fat.

In our investigation we have obtained livers of varying degrees of fatty change and the corresponding gall bladder bile from a number of cases, shortly after death, and subjected both to analysis for fat,—the liver by extraction with hot alcohol and ether, and the bile by the Kumagawa-Suto method for fat estimation, in which the total fatty acid and cholesterol content is determined after saponification. This method gives lower results for the lipoid content of the bile than those usually reported in the literature, partly because of the loss of the glycerol radicle of the fats and the non-lipoidal elements of lecithin in the saponification, but largely, probably, because of the elimination of certain non-lipoidal constituents of the bile, such as cholic acid and similar substances, which are soluble in ordinary diethyl ether, but insoluble in petroleum ether, the final solvent in the Kumagawa method.

It must be admitted, of course, in view of our imperfect acquaintance with the conditions of the bile in the gall bladder, the amount of reabsorption, and so forth, that a study of the bile found in the gall bladder does not furnish a perfect indication of the condition of the bile as secreted originally by the liver. We know that in the biliary tract the bile becomes concentrated, while some substances, notably mucin, are added, and the question arises whether some fat may not be added and also if some fat may not be lost. In this connection we note that Virchow[5] found that a reabsorption of bile fat takes place from the gall bladder epithelium. He states further that in the fattiest livers he has found no fatty change in

[3] Hoppe-Seyler, F., Physiologische Chemie, Berlin, 1881, 317.
[4] Gilbert A., and Jomier, J., *Arch. de méd. expér. et d'anat. path.*, 1905, xvii, 1.
[5] Virchow, R., *Virchows Arch. f. path. Anat.*, 1857, xi, 574.

the epithelium of the gall bladder. On the other hand, we ourselves have several times observed in dogs fatty changes in the gall bladder epithelium when the liver itself was not fatty. In spite of these facts, which make it unsafe to attempt to lay down general principles on the basis of evidence from analyses of gall bladder bile, we felt that a study of the liver and gall bladder bile in man, in various degrees of fatty change of the liver, would be of considerable interest, and accordingly undertook the investigation with the expectation of following it up later with experimental studies upon dogs with biliary fistulæ.

In text-figures 1 and 2 and in table I the results are summarized.

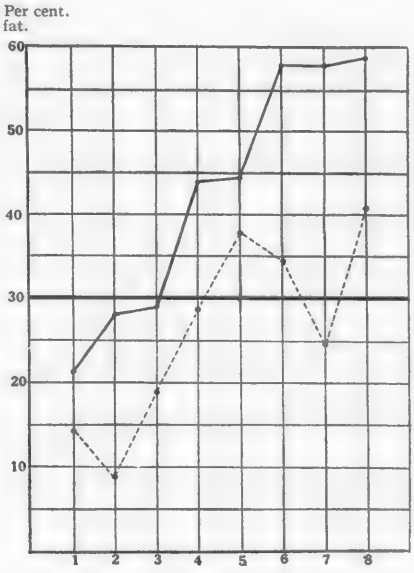
TABLE I.

	Liver.			Bile.			
Case No.	Ratio of total fat to total dry weight.	Ratio of lecithin to fat-free tissue.	Ratio of cholesterol to fat-free tissue.	Amoun in gm.	Total fat in bile per thousand.	Cholesterol in bile per thousand.	Remarks.
1	21.59%	1.05%	1.61%	30	8.6	1.1	Skull fracture and external violence.
2	27.60%	1.43%	2.18%	30	5.0	0.6	Wood alcohol poisoning.
3	28.78%	1.23%	2.86%	15	11.3	2.0	Lye poisoning and bronchopneumonia.
4	44.10%	1.40%	3.20%	15	17.5	1.3	Pulmonary tuberculosis.
5	44.64%	1.23%	2.68%	15	22.8	1.6	Gun shot wound of head. Liver fatty.
6	57.80%	1.49%	3.41%	14	20.4	4.0	Chronic alcoholism.
7	57.80%	———	———	64	14.9	2.3	Chronic morphinism(?).
8	58.66%	1.25%	3.59%	40	24.2	4.0	Alcoholism and chronic nephritis.

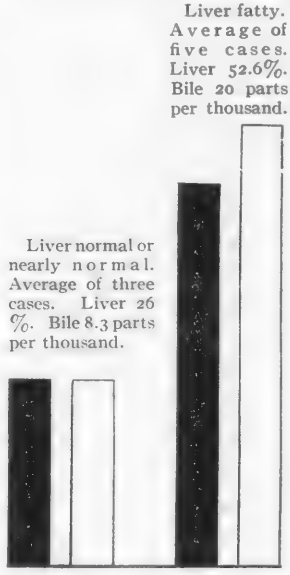
In cases 1, 2, and 3 the liver showed little or no fatty change by analysis or inspection. Cases 4, 5, 6, 7, and 8 were fatty livers. The 30 per cent. line in figure 1 separates the two types. The total fat and lipoid content of the eight livers examined is represented on the solid line in the chart in order of increase, while the fat (fatty acid plus cholesterol) content of the corresponding gall bladder bile is represented on the same ordinate in each case, on a scale convenient for comparison with the liver curve, the broken line constituting the bile curve. As will be noted, the bile curve

is irregular in character and does not correspond exactly with the liver curve, but the chart shows that on the whole the gall bladder bile of the fatty livers averages higher in fat than that found in the gall bladders of the livers which were not fatty.

This fact is illustrated more strikingly by text-figure 2. The normal or nearly normal livers average 26 per cent. fat, and the



TEXT-FIG. 1.——Liver. One space on the ordinate = 5 per cent. of dry weight soluble in ether. ..... Bile. One space = 3 parts of fatty acid + cholesterol per 1,000 parts of bile.


Liver fatty. Average of five cases. Liver 52.6%. Bile 20 parts per thousand.
Liver normal or nearly normal. Average of three cases. Liver 26 %. Bile 8.3 parts per thousand.

TEXT-FIG. 2. Comparison of fat content of liver and corresponding gall bladder bile of livers represented above the heavy line with those represented below the line in text-figure 1. Fat content of liver expressed in per cent. of dry weight soluble in ether. Fat content of bile expressed in parts of bile per 1,000.

corresponding gall bladder biles 8.3 parts per thousand. The figures are represented by columns of equal height, and columns representing the average fat content of the fatty livers and their gall bladder biles are placed beside them for comparison, the second

liver column, representing an average fat content of 52.6 per cent. of the dry weight, being drawn to the same scale as the first liver column, and the second bile column, representing an average of 20 parts per thousand, being drawn to the same scale as the first bile column. A striking parallelism is thus seen, which seems to indicate that in general the fat content of the bile secreted by fatty livers is greater than that of the bile from normal livers, in spite of the possible objections noted above, in view of which, moreover, an absolute parallelism could not be expected. In table I the figures for the analyses and the causes of death are given for each case.

It was thought of interest to determine the lecithin and cholesterol. The former was estimated by the method of Koch and Woods,[6] and the latter by the method of Corper,[7] which was found to yield excellent results and closely agreeing duplicates. As is usually found, the ratio of the lecithin to the dry, fat-free tissue is nearly a constant one, lecithin making up a fairly definite proportion of the cell protoplasm, in both normal and fatty livers, the increased fat of the latter being chiefly infiltrated body fat. A similar relation might be expected to hold for the cholesterol, but our figures show an increase in the amount of this substance accompanying the infiltration of adipose tissue fat.

We wish to express our indebtedness to Dr. E. J. Witzemann for the determination of the dry weight of the liver samples, which was always made immediately after the autopsy.

[6] Koch, W., and Woods, H. S., *Jour. Biol. Chem.*, 1905–06, i, 203.
[7] Corper, H. J., *Jour. Biol. Chem.*, 1912, xii, 197.

SOAPS AS FERMENT-INHIBITING AGENTS.

Studies on Ferment Action. X.*

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The use of the antitryptic index of the blood as a clinical test, particularly in cancer, and the pathological conditions in which ferment action appears to be held in abeyance, especially in caseation in tuberculosis and syphilis, in anemic infarcts, and in the exudate in lobar pneumonia, make the study of ferment-inhibiting substances of great importance. Many substances are considered to have the property of inhibiting the activity of enzymes, but in this paper we shall refer chiefly to the proteolytic enzymes acting in an alkaline medium.

Trypsin and leucoprotease lose their activity when heated at temperatures above 60° C., though according to Salkowski (1) trypsin when dry can be heated to 160° C. without being destroyed. Strong acids and alkalies quickly destroy trypsin, but Chittenden and Cummins (2) found it much more resistant to these agents in the presence of protein. Kudo (3) states that all the mineral acids are active in this respect, sulphuric acid being active in a dilution of one in a thousand, while the organic acids are much less so. Weiss (4) reports that while 0.05 per cent. sodium chloride increases the activity of trypsin, 10 per cent. decreases it. Most of the writers who have investigated the subject find that chloroform, toluol, and thymol inhibit the action of trypsin to a slight degree, but the necessity of a preservative in their experiments caused most workers to ignore this slight inhibition. Bayliss (5) and Abderhalden and Gigon (6) found that some of the products of tryptic activity possess the property of inhibiting the action of the ferment, the free amino acids being more active than the polypeptids. It has been known for several years that blood serum is capable of inhibiting the proteolytic power of trypsin, but in spite of the work of Camus and Gley (7), Charrin and Levaditi (8), Landsteiner (9), and others, we are still ignorant concerning its nature and significance.

In the course of our studies on ferment action we observed that soaps of certain fatty acids were capable of inhibiting the action of trypsin and leucoprotease. This observation seemed to be of importance, as soaps of a similar nature are present in the body, and the possibility of their acting in the same manner

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caused us to make a more careful study of the general phenomena relating to the inhibition of ferment activity by these substances.

Neumann (10), while studying the influence of a fat-rich diet on the digestion of infants, observed that sodium oleate inhibited the action of trypsin. For most of his work he used the Fuld-Gross technique. In some of our preliminary experiments we used the same technique, but soon discarded it in favor of the one which will be described later. According to the Fuld-Gross method the mixture of ferment and substrate is incubated for two hours and then made slightly acid by adding a few drops of a solution containing 5 per cent. acetic acid in 50 per cent. alcohol. The amount of digestion is determined by the degree of cloudiness, the more complete the digestion the clearer the contents of the tube after acidifying. Subsequent experience convinced us of the unreliability of this method, particularly when dealing with lipoidal substances. The acidifying not only coagulates the undigested protein, but also causes the lipoidal substances, particularly the fatty acids, to separate as flocculi, a condition which makes it impossible to determine the extent of proteolysis. Neumann added an excess of alcohol to cause a solution of the precipitated acids and thus solved one difficulty; but he added another by diluting the protein to such a degree that it became a problem to determine the extent of proteolysis.

Two ferments, trypsin and leucoprotease, were used in our work. The trypsin was obtained by extracting commercial pancreatin with N/50 sodium carbonate. After extraction for twenty-four hours, the solution was filtered, and the trypsin in the filtrate precipitated by the addition of nine volumes of alcohol to which sufficient acetic acid had been added to make the whole mixture slightly acid to litmus. This extraction and precipitation was repeated three times. The final product was much stronger than the original and the amount needed to cause complete digestion in the control tubes did not contain sufficient nitrogen to give a definite color when Nesslerized. The leucoprotease was prepared from human pus in the same manner, but the amount required to cause complete digestion in the control tubes contained more nitrogen than the concentrated trypsin. In each experiment the amount of nitrogen was determined in the control tubes and proper correction made in calculating the amount of total incoagulable nitrogen.

Edestin was used as a substrate for several experiments, but later it was discontinued in favor of a 1 per cent. casein solution, the latter being much more soluble in an alkaline medium, and in addition it was noted in precipitating the coagulable protein that a slight excess of acid caused the edestin precipitate to redissolve. After being incubated for the necessary time, the contents of the tubes

were acidified with a mixture containing 10 per cent. glacial acetic acid and 20 per cent. sodium chloride, and the tubes placed in boiling water for five to ten minutes. The mixtures were then filtered through kaolin and the incoagulable nitrogen was determined by the method recommended by Folin (11). The amount of nitrogen found in the control tubes was subtracted from each of the others and the results were given in percentages of total digestion.

The time allowed for incubation was not the same in all experiments. In our preliminary work we found that the concentrated trypsin, prepared as described above, deteriorated rapidly when once dissolved. For this reason the trypsin solution was prepared immediately before being used, one cubic centimeter representing 0.002 of a gram of trypsin. Several tubes containing only casein and trypsin were placed in the incubator at the same time as the tubes or flasks containing the mixtures of soaps. One of the tubes containing casein and trypsin was removed after one hour and tested. After that the other tubes were removed at intervals of fifteen minutes, also tested, and as soon as digestion was found to be complete the remainder were immediately removed, acidified, and boiled.

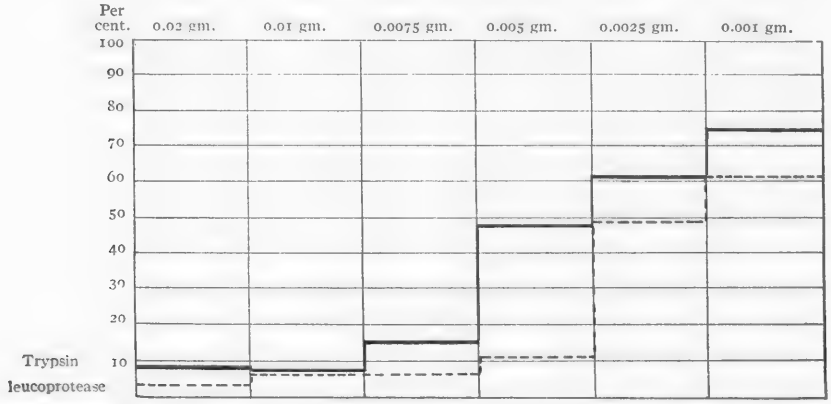
The protocols are not given for each experiment as the charts present the results more clearly. Table I gives the number of controls used in each experiment, and also the amount of nitrogen obtained by complete digestion and as a result of the inhibiting action of the soaps.

In most instances we did not know the identity of the acids used in the preparation of the soaps, but the isolation and identification of these with further work on their ferment-inhibiting action is now under way. The three supposedly pure soaps were Kahlbaum's preparations, and consisted of sodium oleate, sodium palmitate, and sodium stearate. On examination we found that the sodium oleate had become saturated, making it useless as an oleate, though it was used in several experiments as a representative of the saturated fatty acids.

The majority of the soaps were obtained by saponifying linseed, olive, cod-liver, hempseed, and castor oils, as these oils are known to contain a large number of unsaturated fatty acids. The oils were saponified with alcoholic potash, and the soaps extracted re-

peatedly with petroleum ether. The acids were then liberated by hydrochloric acid, taken up in ether, the ether evaporated, and the acids again saponified. This process was repeated and the acids were preserved pure until needed. The soaps used in the work were rarely more than two or three days old.

Later when it was found that the unsaturated fatty acids were the active inhibiting agents, the lead soap-ether method of obtaining these in higher concentrations was used. The separation of the saturated and unsaturated fatty acids by this method is not complete, but is sufficiently so to enable us to show marked difference between the action of the ether-soluble and insoluble fractions. In

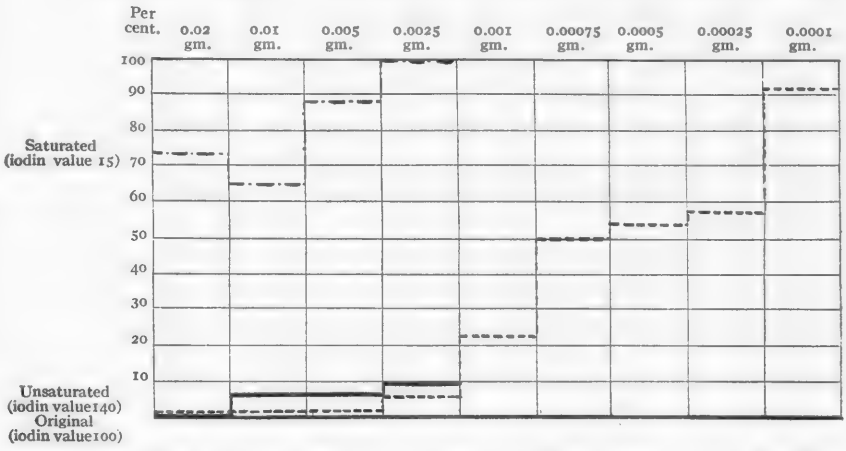


TEXT-FIG. 1. Effect of sodium oleate on tryptic and leucoproteolytic digestion.

nearly every instance the iodin value of the preparations was determined before they were used.

One of the first soaps investigated was that prepared from olive oil. Its influence on leucoprotease and trypsin is shown in text-figure 1. In this experiment the soap and ferment were mixed, incubated for thirty minutes at 37° C., and the casein was then added. This procedure was adopted in all the subsequent experiments. Text-figure 1 shows that even 0.001 of a gram of the soap is sufficient to affect materially the activity of both ferments. In this and in subsequent charts the control tubes showing 100 per cent. of digestion are not given.

Efforts were then made to determine if the inhibiting action displayed by these soaps was due to the saturated or unsaturated fatty acids present. Soaps from nearly all the oils were tested in a similar manner, but as uniform results were obtained, we shall give only one example. Text-figure 2 shows the results obtained with



TEXT-FIG. 2. Effect of saturated and unsaturated linseed soaps on tryptic digestion.

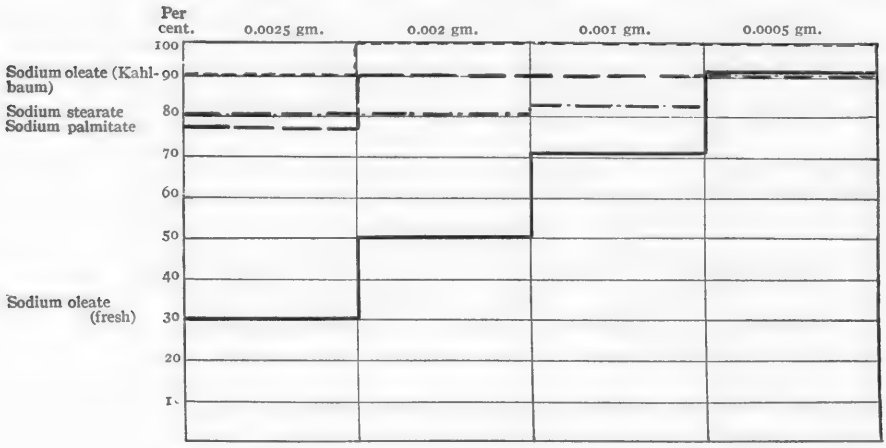
the soap prepared from linseed oil. The ether-lead soap method was used in separating the groups of acids. The chart indicates that the unsaturated fatty acids are the active inhibiting agents. It is well known that the ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, and this probably explains why the saturated fraction, the ether-insoluble lead soaps, still retained about one fourth of its inhibiting action, whereas the unsaturated fraction, the ether-soluble lead soap, now caused complete inhibition.

During the progress of the work we obtained three of Kahlbaum's supposedly pure soaps, sodium oleate, sodium palmitate, and sodium stearate. The results obtained with these three soaps and with one prepared by ourselves from olive oil are shown in text-figure 3. The iodin value of the linseed oil soap prepared by us was 90.

Our previous experiments indicated that the soaps of all the unsaturated fatty acids acted as inhibiting agents. We were therefore

surprised to note that the Kahlbaum sodium oleate was inactive. The solution of the problem was soon found, the sodium oleate had no iodin value; in other words, the oleic acid, through age or some other cause, had become saturated. The sodium soaps of palmitic and stearic acids, as we had anticipated, had no influence on either trypsin or leucoprotease.

All our experiments so far indicate that the inhibiting agents present in the soaps are the unsaturated fatty acids. The question therefore naturally presents itself: Can we by a saturation of these



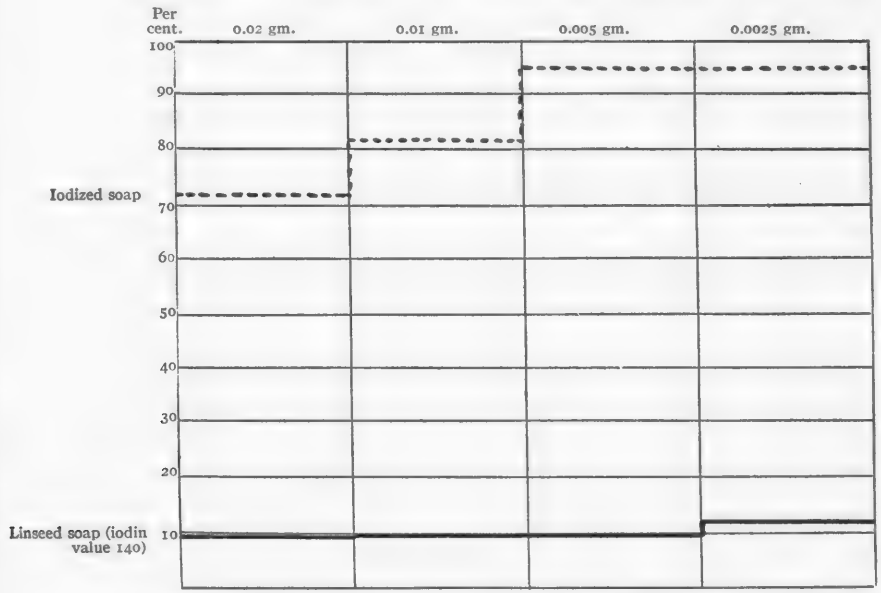
TEXT-FIG. 3. Effect of sodium oleate, sodium stearate, and sodium palmitate on tryptic digestion.

free bonds with iodin, for instance, destroy the inhibiting action and cause them to act like the soaps prepared with stearic, palmitic, and other saturated acids?

The results of one of the experiments made to enlighten us on this point are given in text-figure 4. This experiment was conducted with the ether-soluble lead soap fraction. To five cubic centimeters of a 1 per cent. solution of the soap were added 0.05 of a gram of iodin and a few crystals of iodide of potassium, and the mixture was permitted to stand over night. The following morning it was shaken repeatedly with chloroform, until fresh portions of the

latter remained clear, showing that there was no more free iodin. Text-figure 4 shows the action of the unsaturated soap before and after it was treated with iodin. Repeated tests had already shown that the small amount of iodin which might still remain would have a slight inhibiting action. This would explain why the soap still had some inhibiting action, though it may also be due to the fact that the acids were not completely saturated. The results of saturating the free carbon bonds are so obvious as to call for no comment, and also explain why the commercial sodium oleate was inactive.

Similar experiments made with sodium soaps of unsaturated fatty acids obtained from other sources indicated that the inhibiting



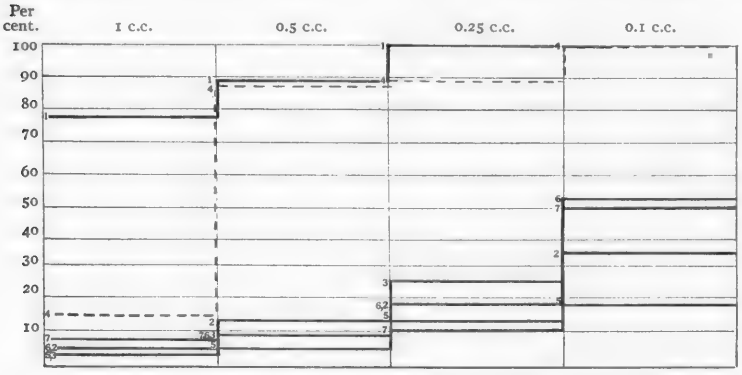
TEXT-FIG. 4. Effect of saturation of unsaturated linseed soaps with iodin.

action was due to the degree of unsaturation and that it could be almost entirely removed by saturation with iodin.

Our next experiments were made to determine whether the activity of the various soaps was dependent upon the number of unsatu-

rated bonds, as determined by their iodin values, or whether certain ones containing an equal number of unsaturated bonds were more active.

In this experiment soaps prepared from linseed oil, hempseed oil, castor oil, olive oil, croton oil, and cod-liver oil were used. After their respective iodin values had been determined, they were made up in such strengths that one cubic centimeter of each had the same iodin value; thus croton oil soap having less than half the value of the linseed oil soap was made up twice as strong. Text-figure 5



1 = saturated linseed soap.	Iodin value 15.	Solution 5.0	per cent.
2 = sodium oleate.	Iodin value 53.	Solution 1.9	per cent.
3 = croton soap.	Iodin value 55.	Solution 1.8	per cent.
4 = castor oil soap.	Iodin value 60.	Solution 1.7	per cent.
5 = hempseed soap.	Iodin value 100.	Solution 1.0	per cent.
6 = cod-liver oil soap.	Iodin value 90.	Solution 1.1	per cent.
7 = linseed soap.	Iodin value 135.	Solution 0.74	per cent.

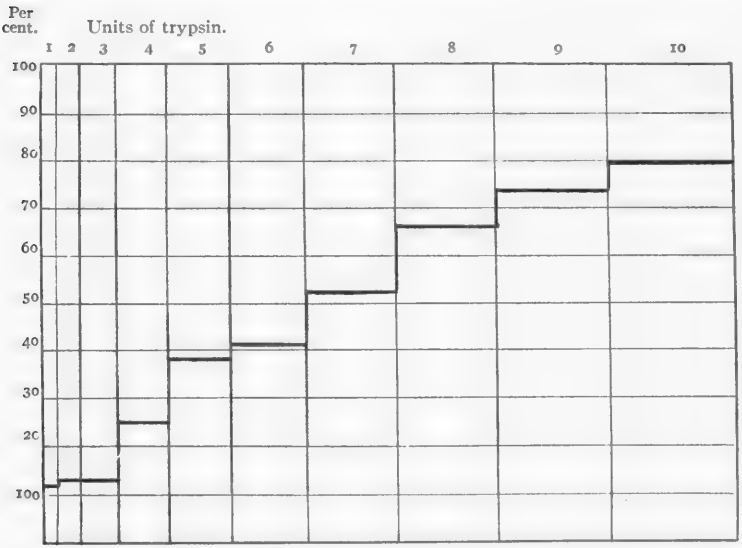
TEXT-FIG. 5. Relation of iodin value to inhibiting action of soaps on tryptic activity.

gives the results of this experiment. The lines shown on the chart indicate, with one exception, that the activity of the soaps is in proportion to their iodin values.

Here we have the first indication that the unsaturated fatty acids are not equally active as inhibiting agents, for the soap prepared from castor oil appears to be considerably less active. The inhibition obtained with one cubic centimeter was almost as great as that noted with the other soaps, but subsequent dilution proved it to be

much less active. With the exception of that prepared from linseed oil, the soaps used in this experiment were not obtained by the ether-lead soap method, and so their iodin values were low. It is possible that some of the unsaturated fatty acids are much more active than others in this respect. In fact, a part of our recent work indicates that those obtained from a certain source are much more active than any of those we have just discussed.

In the preceding experiments it has been demonstrated that 0.005 of a gram of the linseed oil soap was sufficient to prevent the action of the standard amount of trypsin. It was also found, in the standard proportions of ferment and substrate used, that the inhibiting action was still present in tubes containing much smaller amounts. In fact complete digestion was first obtained in the tube containing



TEXT-FIG. 6. Effect of increasing amounts of trypsin with single unit of soap.

0.0001 of a gram. In our next experiment we wished to find out if the smallest amount of soap producing nearly complete inhibition with the standard amounts of trypsin and casein would have any influence on larger amounts of the ferment. Text-figure 6 shows

the influence of the soap on increasing amounts of the ferment. 0.1 of a cubic centimeter of the ferment solution caused complete digestion in the control tube. The demonstration that such a small amount of soap, 0.005 of a gram, was able to affect materially the action of ten times the standard amount of ferment, indicates the great activity of these substances as inhibiting agents.

In the previous experiments we have shown that the unsaturated fatty acids inhibit proteolysis in an alkaline medium and that the degree of inhibition bears a close relation to their iodin values, but these experiments do not give us much information as to how the inhibition is effected. Is it due to a direct and permanent binding of the ferment with the unsaturated fatty acids, or to simple inhibition of ferment action, the ferment itself not being destroyed? If there is a true binding it would be difficult to extract the ferment after incubating with soap, but it might be done if the inhibition were due to some other cause.

Our next experiment was planned with the hope that some information might be obtained as to the nature of the inhibiting action. In this experiment the soaps of the saturated and unsaturated acids separated by the ether-lead soap method were mixed with the ferment and incubated for thirty minutes. The mixtures were then made slightly acid with hydrochloric acid and extracted with ether. After having removed the ether, the solution was made slightly alkaline, the casein added and again incubated. One control tube of ferment without soap was treated in the same manner so as to rule out the effect of the acid and ether. In this experiment we wished to find out if the soap simply inhibited the action of the ferment or destroyed it. Table I gives the results of this experiment.

TABLE I.

	Trypsin.	1 per cent. unsaturated linseed soap, iodin value 130.	1 per cent. saturated linseed soap, iodin value 15.	Sodium chloride solution.	1 per cent. casein.	Total incoagulable nitrogen.	Digestion.
1	0	0	0	3.0 c.c.	2.0 c.c.	0.04 mg.	2 %
2	0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.66 mg.	100 %
3	0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.10 mg.	6 %
4	0.2 c.c.	0	0.5 c.c.	2.3 c.c.	2.0 c.c.	1.30 mg.	80 %
5	0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.50 mg.	90 %
6	0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.20 mg.	12 %
7	0.2 c.c.		0.5 c.c.	2.3 c.c.	2.0 c.c.	1.66 mg.	100 %

The first four tubes in the table are the untreated controls. The first tube shows the percentage of incoagulable nitrogen in the casein alone; the second, the total nitrogen in complete digestion; the third and fourth show the influence of the saturated and unsaturated soaps on tryptic activity. Tubes 5, 6, and 7 show the influence of acid and ether extraction. Tube 5 contained no soap but was treated in the same way as Nos. 6 and 7 to make certain that the method used had not destroyed the ferment. Tube 6 containing the unsaturated soap shows that the ferment action is almost inhibited, while in tube 7 containing the saturated soap there is no inhibition. In other words, incubating the ferment with soaps of the unsaturated fatty acids for thirty minutes causes its destruction, the removal of the acids after incubation not being sufficient to reactivate the ferment, while the ferment present in the tube containing the saturated acid, though treated in the same manner, is not destroyed.

The results obtained in this preliminary study of the action of the soaps prepared from the unsaturated fatty acids suggest that they may play an important part in the body, particularly in certain pathological processes. Whether this inhibition of ferment action is due to a binding of the ferment with the unsaturated carbon atoms, or whether to some physical condition brought about by the presence of the soap, remains to be determined. If due to some physical condition brought about by the presence of the soap, similar results ought to be obtained with the saturated fatty acids, but we have demonstrated that the latter are inactive in this respect. In addition, the experiments conducted with soaps saturated with iodin show that the presence of unsaturated bonds are necessary for the development of this property. Further proof that this action is due to the degree of unsaturation is afforded by text-figure 5, which indicates that the activity of the soaps is proportionate to their iodin value, while the soaps having no iodin value, sodium stearate and sodium palmitate, are inactive.

SUMMARY.

1. Sodium soaps prepared from olive oil, croton oil, cod-liver oil, linseed oil, etc., have the property of inhibiting the action of trypsin and leucoprotease.

2. The activity of these soaps is dependent upon the degree of unsaturation of the fatty acids and is in proportion to their iodin value.

3. Saturation of the acids with a halogen (iodin) causes a loss of this property.

4. Soaps of the saturated fatty acids tested do not have this influence on ferments.

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FERMENT-INHIBITING SUBSTANCES IN TUBERCLE BACILLI.

Studies on Ferment Action. XI.*

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A great deal of work has been done in the attempt to explain the processes that lead to the production of caseation in tuberculosis. By some it has been ascribed to anemia, the tubercle being avascular, and by others to the action of toxins derived from the tubercle bacilli.

Auclair (1) believes that the caseation is due to specific toxins of the nature of fat, which are soluble in ether, chloroform, benzine, and xylol. He treated tubercle bacilli with one or more of the above agents, evaporated the solvents, and suspended the extracted substances in water. These emulsions, when injected into the subcutaneous tissues of animals, produced typical caseous abscesses. When injected into the trachea of guinea pigs, caseous areas appeared in the lungs.

Schmoll (2) analyzed caseous material and found it almost free from proteoses, which indicates that autolysis is slight; but he does not explain the lack of autolysis. The work of Schmoll has been confirmed by others, and we have obtained similar results.

Anemia due to the occlusion of the blood vessels may be an important factor in causing caseation in the chronic forms of tuberculosis where the areas are surrounded by a layer of connective tissue. However, in acute caseous pneumonia in which there is a catarrhal exudate rapidly becoming caseous, some other explanation appears to be necessary. In such an exudate there are numerous cells known to contain ferments, yet these die and disintegrate, and, as far as can be determined, the ferments remain inactive. We must therefore look for some other factor to explain the process of caseation, and this we believe we have found in the tubercle bacillus, a ferment-inhibiting substance. We observed two years ago that the

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antitryptic action of sera was lost or greatly decreased when the sera were preserved with chloroform. This observation suggested to us the possibility that the antiferment properties of the blood were due to substances of the nature of lipoids, and the lack of autolysis in caseation and in anemic infarcts to substances of a similar nature.

With this hypothesis we decided to study the influence of the ether-soluble substances of tubercle bacilli on proteolysis. It has been shown by numerous investigators that the ether-soluble substances constitute 25 to 35 per cent. by weight of the bacilli, and of these extracts about 17 per cent. are composed of fatty acids. Both human and bovine tubercle bacilli were used in the work.[1]

Our first experiments were conducted with ether- and alcohol-soluble substances obtained from the tubercle bacilli. After the solvent had been evaporated, the material remaining was taken up in methyl or ethyl alcohol and suspended in a 0.9 per cent. solution of sodium chloride. In some experiments the emulsion was used just as prepared; in others the alcohol was first evaporated at low temperatures. The emulsion, in various dilutions, was then mixed with trypsin, incubated for thirty minutes, and the casein added. As far as we could determine there was no inhibition of ferment action.

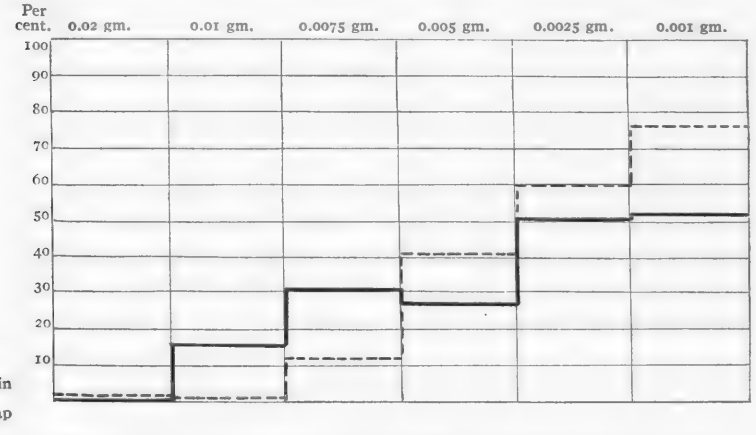
In the preliminary experiments we used the Fuld-Gross technique, but, for reasons given in our preceding paper (3), we soon discarded it in favor of the following. After incubating the mixtures for a certain period of time, they were acidified with a solution containing 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes, filtered through kaolin to remove the coagulated protein, and the incoagulable nitrogen was determined according to the method recommended by Folin (4).

This experiment failed to demonstrate the presence of an inhibiting agent. This may have been due to the agent being in an emulsion and not a solution, thus preventing its coming into intimate con-

[1] We wish to thank Dr. Hitchens, of Glenolden, Pa., and Dr. Grund, of the New York Board of Health, for supplying us with large amounts of human and bovine tubercle bacilli.

tact with the ferment. We then fractionated the extracted material so that at least a part, the fatty acids, might be prepared in such a manner that they could be brought into solution. The material was dissolved in ether and precipitated with acetone. The acetone fraction was evaporated to dryness on the water bath and the resulting substance saponified with alcoholic potash. The soap was dissolved in water and repeatedly extracted with petroleum ether in order to remove any unsaponifiable matter. The acids were then liberated with hydrochloric acid, taken up with ether, washed with water, and resaponified.

The ferments were prepared and standardized according to the

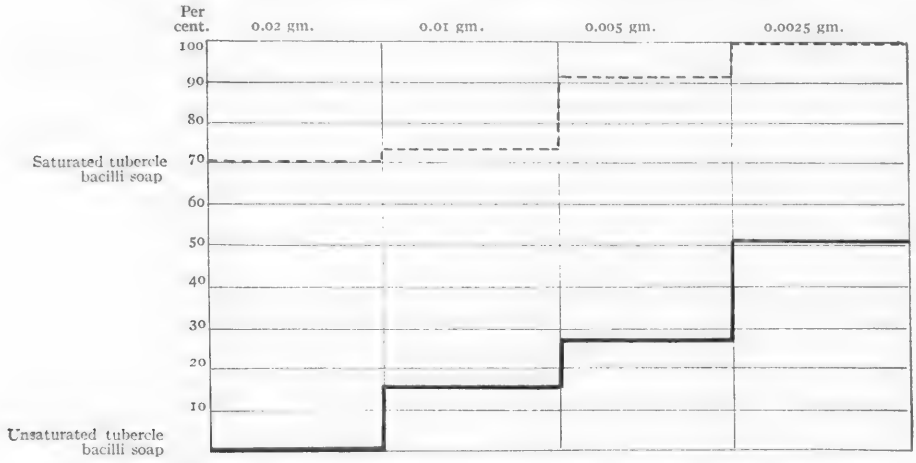


TEXT-FIG. 1. Effect of soaps of tubercle bacilli and sodium oleate on tryptic digestion.

method described in our previous paper (5). The soaps, in 1 per cent. solutions, were mixed with trypsin in varying amounts, incubated for thirty minutes, and the casein was then added. After the final incubation of two hours, the coagulable protein was removed and the incoagulable nitrogen determined. Text-figure 1 shows the results obtained with the soap prepared from extractives of the tubercle bacilli. A soap prepared from olive oil was used as a control. The dotted and heavy black lines indicate the percentage of digestion present in the tubes containing soaps as compared with the controls containing trypsin and casein alone.

The results of this experiment show that in the final dilutions the soap prepared from tubercle bacilli is more active as an inhibiting agent than the one prepared from olive oil. The iodin value of the soap prepared from the tubercle bacilli was 24, of the olive oil soap 50. The relation that the iodin value of the soaps bears to their activity as inhibiting agents was discussed in our previous report. It will be dealt with here in relation to the one prepared from tubercle bacilli.

In the previous paper (5) on the influence of soaps of the fatty acids on inhibition of ferment action, we demonstrated that the unsaturated fatty acids were the active agents, while the saturated fatty acids exerted but little if any inhibiting action. We were unable to obtain a complete separation of the saturated from the unsaturated acids and so could not remove entirely the inhibiting



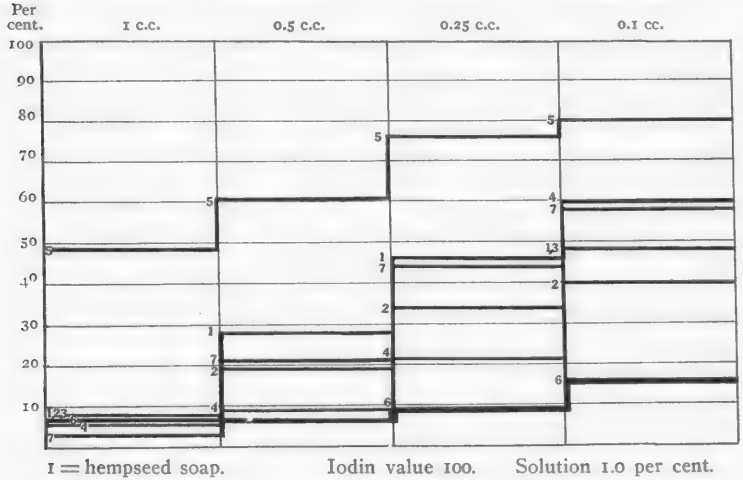
TEXT-FIG. 2. Effect of saturated and unsaturated soaps of tubercle bacilli on tryptic digestion.

action, but the differences in their activity following their partial separation by the ether-lead soap method were so striking as to be conclusive. The soaps of the saturated fatty acids tested, sodium stearate and sodium palmitate, were inactive.

We undertook to determine if the same is true of the soaps pre-

pared from tubercle bacilli. Lead soaps were prepared according to the usual method. The acids were set free with hydrochloric acid, washed, resaponified with sodium alcoholate, and evaporated to dryness. Text-figure 2 gives the results obtained with soaps prepared from saturated and unsaturated fatty acids.

The results obtained with the ether-soluble and insoluble fractions are as conclusive as those described in the preceding report for other soaps. The ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, but the separation is sufficient to demonstrate which is the active fraction.



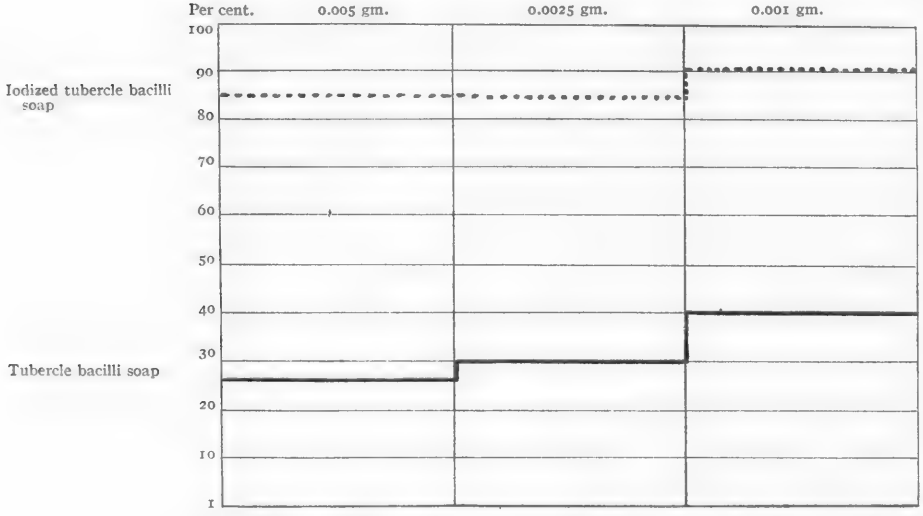
1 = hempseed soap.	Iodin value 100.	Solution 1.0 per cent.
2 = linseed soap.	Iodin value 100.	Solution 1.0 per cent.
3 = croton soap.	Iodin value 61.	Solution 1.6 per cent.
4 = olive oil soap.	Iodin value 44.	Solution 2.2 per cent.
5 = castor oil soap.	Iodin value 35.	Solution 3.0 per cent.
6 = tubercle bacilli soap.	Iodin value 25.	Solution 4.0 per cent.
7 = cod-liver oil soap.	Iodin value 92.	Solution 1.1 per cent.

TEXT-FIG. 3. Effect of soaps of tubercle bacilli and various other soaps on tryptic digestion.

We reported previously that, with one exception, the activity of the soaps of the unsaturated fatty acids as inhibiting agents was proportionate to their iodin value. We did not include in the experiment described the soap prepared from the extractives of

the tubercle bacilli. This is shown in text-figure 3 in comparison with the other soaps which we have been using. After the iodin value of the respective soaps had been determined, they were made up in such strengths that one cubic centimeter of each had approximately the same iodin value.

A study of text-figure 3 shows that of all the soaps investigated the one prepared from tubercle bacilli is the most active in preventing enzyme action. We had already observed that it was as active as the other soaps in 1 per cent. solutions and were not surprised to find it more so when made up according to its iodin value.



TEXT-FIG. 4. Effect of saturation of unsaturated soaps of tubercle bacilli with iodin.

The method used by us in extracting the acids from the bacilli and in preparing the soap does not give any indication of the quantity originally present. On the contrary, it offered every opportunity for the unsaturated acids to become saturated, thus lowering its iodin value and reducing its activity as an inhibiting agent.

In the previous report we showed that the activity of the soaps of the unsaturated fatty acids could be destroyed by saturation with iodin. The soaps so treated did not become entirely inactive,

but this may have been due either to the presence of some free iodin or to incomplete saturation. It is of importance to determine if the soaps prepared from tubercle bacilli are similarly affected when treated with iodin. To five cubic centimeters of a 1 per cent. solution of the soap was added 0.05 of a gram of iodin, and a few crystals of iodide of potassium, and the mixture was permitted to stand over night. The following morning it was shaken repeatedly with chloroform, until fresh portions of the latter remained clear, showing that there was no more free iodin. Text-figure 4 shows the effect of saturating the soap with iodin.

The soap used in this experiment was old and therefore not very active, but the effect of treatment with iodin was evident. The experiment was repeated several times and a similar result obtained in each instance.

Numerous chemicals have been studied in the attempt to find one that will be effective as a therapeutic agent in tuberculosis. Such an agent, in order to be of value, must combine with some constituent of the tubercle bacillus, and our experiments indicate that iodin as a component of the chemical substance may be the means of bringing about such a combination.

We do not know the nature of the unsaturated fatty acids exerting the inhibiting action, but their isolation and identification and further work on their inhibiting action are now under way.

Wells (6) obtained an average of 0.001 of a gram of fatty acids to one gram of caseous matter, which indicates the small amount of soap present in this substance. This finding has little bearing on the relation of the fatty acids of tubercle bacilli to caseation, as the influence of the soaps depends on the amount of ferment present and not on the quantity of protein. That small amounts of the soap are capable of inhibiting enzyme action is shown in text-figure 1, where it was found that in the tube containing 0.001 of a gram of soap the digestion was only half that obtained in the control containing no soap. The influence of soaps as inhibiting agents was well shown in our previous report in which it was demonstrated that the smallest quantity completely inhibiting the standard amount of trypsin materially influences the action of ten times that amount of ferment. In

addition, our experiments indicate that the ferments are destroyed as a result of combining with the soaps.

The soaps are probably formed from the neutral fats of the bacilli. The lipases hydrolyze the fats and the acids are then saponified. In partial support of this view we have the work of Klotz (7) who demonstrated lipases in tuberculous pus, and of Bergel (8) who states that the ferment is present in the lymphoid cells of tubercles, while Corper (9) found lipases in tubercle bacilli.

The demonstration that tubercle bacilli contain ferment-inhibiting substances brings us one step nearer a rational explanation of caseation in tuberculosis. The subject will be discussed more fully in a subsequent report.

CONCLUSIONS.

1. Tubercle bacilli contain unsaturated fatty acids which, when saponified, have the property of inhibiting the action of trypsin and leucoprotease.

2. In proportion to their iodin value these soaps are more active as inhibiting agents than the soaps prepared from linseed, olive, and cod-liver oils.

3. The activity of the soaps is dependent on the presence of unsaturated carbon bonds.

4. Saturation of the soaps with iodin destroys their inhibiting action.

5. Soaps probably play an important part in the production of the condition known as caseation in tuberculosis.

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A FURTHER STUDY OF NITROGEN RETENTION IN THE BLOOD IN EXPERIMENTAL ACUTE NEPHRITIS.*

By HOWARD T. KARSNER, M.D., and W. DENIS, Ph.D.

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In a previous communication[1] we reported the results of the study of experimental acute nephritis in the cat produced by uranium nitrate, by potassium chromate, and by cantharidin. It was shown that these forms of nephritis result in moderate but distinctive retention in the blood of non-protein and urea nitrogen; that from the anatomical point of view the almost purely tubular forms of nephritis result in moderate retention of nitrogen; and that the additional anatomical involvement of the glomerulus leads to a somewhat greater retention. Since the publication of this report the same methods have been applied in other studies. Frothingham, Fitz, Folin, and Denis[2] have shown that the nitrogen retention and phenolsulphonephthalein excretion are parallel and in accordance with the degree of anatomical lesion in uranium nephritis of the rabbit, and that nitrogen retention lags somewhat behind the diminished excretion of the dye. Farr and Austin[3] have shown that in human cases the appearance of chronic passive congestion of the kidneys leads to no increase in the total non-protein nitrogen; that in chronic nephritis associated with marked albuminuria and edema there is little if any increase; that in chronic nephritis with hypertension

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, December 24, 1913.

[1] Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789.

[2] Frothingham, C., Jr., Fitz, R., Folin, O., and Denis, W., The Relation between Non-Protein Nitrogen Retention and Phenolsulphonephthalein Excretion in Experimental Uranium Nephritis, *Arch. Int. Med.*, 1913, xii, 245.

[3] Farr, C. B., and Austin, J. H., The Total Non-Protein Nitrogen of the Blood in Nephritis and Allied Conditions, *Jour. Exper. Med.*, 1913, xviii, 228.

there is a well marked increase in total non-protein nitrogen with a percentage increase of the ammonia-urea fraction; and that there is a concordance between the appearance of uremia and increase in the total non-protein nitrogen. Folin, Denis, and Seymour[4] have shown that in cases of chronic interstitial nephritis with high blood pressure it is possible by low protein diet to reduce the total non-protein nitrogen practically to normal, and they state that there is "no marked connection between the blood pressure and the degree of retention, indeed it is doubtful whether there is any connection." They also found no close connection between phenolsulphonephthalein excretion and nitrogen retention, presumably because the latter is so much under the influence of diet.

The purpose of the present communication is to present observations of other varieties of experimental acute nephritis than those presented in the first publication, thus including the forms produced by a specific hemolytic immune serum, by sodium arsenate, by diphtheria toxin, and by tartaric acid. The same technique was employed as in the preceding study, and, in addition, as often as possible two determinations of the normal blood were made in each experiment. Unsuccessful attempts were made to produce an experimental nephritis of the cat by the use of rattlesnake venom.[5] A cat weighing 2,500 grams died in thirty-six hours from a hypodermatic injection of 0.010 of a gram of dried venom; a cat weighing 2,200 grams survived an injection of 0.009 of a gram of dried venom; and a cat weighing 3,300 grams survived an injection of 0.013 of a gram of dried venom. None of the animals showed any retention of non-protein nitrogen, and histologically their kidneys showed nothing that could be interpreted as nephritis.

[4] Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press). Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

[5] The dried venom of *Crotalus adamanteus* was given us by Dr. Joseph MacFarland of Philadelphia.

IMMUNE SERUM NEPHRITIS.

It has been pointed out by Pearce and Eisenbrey[6] that the nephritis produced by a hemolytic immune serum differs from that produced by a specific nephrotoxic serum principally in that the former shows more marked glomerular change histologically. Physiologically, however, there is no notable difference between the two, both showing the vascular reactions of a tubular nephritis. Because of the greater ease of preparation, hemolytic immune serum was used in the present study. In the cat there is, in the early stage of this variety of nephritis, moderate cloudy swelling of the tubular epithelium most noticeable in the distal convoluted tubules associated with moderate swelling of the capillary endothelium of the tuft and the appearance of much albuminous precipitate in the subcapsular space. Hyaline and granular casts are frequent in the ascending loops of Henle, but the picture throughout is obscured by the bile and hemoglobin staining of the tissues. After the disappearance of this staining the glomerular change is somewhat more marked and the degeneration of the distal convoluted tubules persists, in diminishing degree of severity, up to twelve days, the longest period of observation.

Cat 15.—Weight 1,650 gm. Given intravenously 0.8 c.c. of specific hemolytic immune serum (about 0.5 c.c. per kilo). Bled on the 2d, 3d, 4th, 8th, 10th, and 12th days. Hemoglobinuria for 24 hours. Albuminuria appeared on the 2d day and continued until the 4th day, but had disappeared on the 8th day. Killed with chloroform on the 12th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (8 dys.)	42 mg.[7]	27 mg.
Before injection (4 dys.)	41 mg.	27 mg.
21 hrs. after injection	52 mg.	35 mg.
45 hrs. after injection	47 mg.	31 mg.
69 hrs. after injection	48 mg.	23 mg.
7 dys. after injection	52 mg.	27 mg.
9 dys. after injection	42 mg.	23 mg.
11 dys. after injection	55 mg.	22 mg.

[6] Pearce, R. M., and Eisenbrey, A. B., A Physiological Study of Experimental Nephritis Due to Bacterial Poisons and Cytotoxic Sera, *Jour. Exper. Med.*, 1911, xiv, 306.

[7] In all cases the figures represent milligrams per 100 c.c. of blood.

Cat 16.—Weight 1,950 gm. Given intravenously 1.5 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Albuminuria and hemoglobinuria on the 2d day. Killed with chloroform on the 2d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 wks.)	39 mg.	26 mg.
Before injection (2 wks.)	39 mg.	25 mg.
24 hrs. after injection	105 mg.	84 mg.

Cat 17.—Weight 2,150 gm. Given intravenously 1.6 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Hemoglobinuria and albuminuria on the 2d day. Found dead on the morning of the 3d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	40 mg.	24 mg.
Before injection (4 dys.)	34 mg.	19 mg.
24 hrs. after injection	80 mg.	45 mg.

It will be seen that a relatively small dose of hemolytic immune serum is capable of producing a slight retention of nitrogen in the blood and that larger doses result in well marked retention of nitrogen with a considerable increase in the urea fraction. Thus a nephritis which shows extensive tubular change histologically and moderate glomerular change, a nephritis which physiologically is tubular in character, produces a moderate degree of nitrogen retention.

ARSENIC NEPHRITIS.

Arsenic produces a nephritis in the cat which in the earlier stages shows histologically only slight glomerular change in the form of albumin in the subcapsular space and an occasional swollen endothelial cell. The tubular epithelium shows a rapidly advancing cloudy swelling, proceeding particularly in the proximal convoluted tubules to marked necrosis; there is much granular and hyaline cast formation. In the later stages (two weeks) the epithelium shows a marked tendency to recovery and the glomerulitis becomes somewhat more distinct but never very severe. This form of nephritis in dogs is primarily vascular in the physiological sense,[8] but in its later stages (three to five days) is more nearly like the tubular forms. Examinations of the urine have shown an increased output

[8] Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., Experimental Acute Nephritis: The Vascular Reactions and the Elimination of Nitrogen, *Jour. Exper. Med.*, 1910, xii, 196.

of nitrogen. The highly fatal character of arsenic intoxication renders the study of the condition somewhat more difficult than in the case of the other nephrotoxic agents.

Cat 18.—Weight 2,400 gm. Given subcutaneously 0.025 gm. of sodium arsenate (about 0.010 gm. per kilo). Bled after 6 hours and on the 2d, 5th, and 6th days. No albuminuria up to the 11th day. On the 11th day given 0.075 gm. of sodium arsenate (about 0.030 gm. per kilo). Bled after 18 hours, after showing slight albuminuria; killed with chloroform after bleeding.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	40 mg.	20 mg.
Before injection (3 wks.)	40 mg.	21 mg.
3 hrs. after 1st injection	46 mg.	28 mg.
30 hrs. after 1st injection	47 mg.	28 mg.
78 hrs. after 1st injection	41 mg.	23 mg.
102 hrs. after 1st injection	50 mg.	30 mg.
18 hrs. after 2d injection	79 mg.	66 mg.

Cat 19.—Weight 2,500 gm. Given subcutaneously 0.075 gm. of sodium arsenate (0.030 gm. per kilo). Bled after 6 hours, at which time moderate albuminuria was found. Found dead at the end of 24 hours.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 dys.)	38 mg.	20 mg.
6 hrs. after injection	40 mg.	28 mg.

Cat 20.—Weight 2,080 gm. Given subcutaneously 0.045 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d, 3d, 5th, 7th, 8th, and 14th days. Albuminuria appeared on the 2d day and continued until the 7th, but had disappeared on the 14th day. Killed with chloroform on the 18th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (19 dys.)	44 mg.	28 mg.
Before injection (2 wks.)	43 mg.	28 mg.
24 hrs. after injection	63 mg.	50 mg.
48 hrs. after injection	55 mg.	39 mg.
96 hrs. after injection	50 mg.	40 mg.
120 hrs. after injection	47 mg.	30 mg.
2 wks. after injection	39 mg.	17 mg.

Cat 21.—Weight 1,850 gm. Given subcutaneously 0.043 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d and 4th days. Albuminuria appeared on the 2d day and continued until death. Animal was found dying on the 5th day, and blood was taken from the posterior vena cava as death occurred.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (6 dys.)	42 mg.	27 mg.
Before injection (5 dys.)	43 mg.	27 mg.
24 hrs. after injection	52 mg.	39 mg.
72 hrs. after injection	125 mg.	98 mg.

Cat 22.—Weight 2,000 gm. Given subcutaneously 0.050 gm. of sodium arsenate (0.025 gm. per kilo). Bled on the 2d, 4th, and 10th days. Albuminuria appeared on the 2d day and continued until death. Killed with chloroform on the 10th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	39 mg.	25 mg.
Before injection (3 dys.)	45 mg.	30 mg.
24 hrs. after injection	95 mg.	68 mg.
72 hrs. after injection	40 mg.	27 mg.
216 hrs. after injection	57 mg.	29 mg.

From the results it will be seen that arsenic nephritis is accompanied by a moderate retention of nitrogen in from twenty-four to seventy-two hours, but that in the early stages, when physiologically it is of vascular type the retention is extremely slight. In the animal with most marked nitrogen retention the changes histologically were almost solely tubular, even at a stage when physiologically the condition is a tubular nephritis. From the point of view of nitrogen retention, the nephritis of arsenic shows the most marked retention at the time when the tubular changes are most prominent.

DIPHTHERIA TOXIN NEPHRITIS.

The nephritis produced by a single toxic dose of diphtheria toxin is essentially an acute glomerular nephritis.[9] There is swelling of the glomerular tuft with slight endothelial proliferation, infiltration of the polymorphonuclear leucocytes, occasionally pyknotic nuclei, albumin in the subcapsular space, occasionally hyaline thrombi, and swelling of the subcapsular epithelium. The tubular epithelium shows moderate cloudy swelling in the earlier cases with the accumulation of a moderate amount of granular material in the lumina of the tubules. Later there is more marked degeneration and small areas of actual necrosis. The change affects all the tubular epithelium, but most markedly that of the proximal convoluted tubules.

For the sake of uniformity the dose is given in units. It has been shown by Paton, Dunlop, and Macadam[10] that there is an in-

[9] The toxin used was furnished and titrated by Dr. Theobald Smith.

[10] Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

creased output of nitrogen following the administration of diphtheria toxin, which they believe to be due to the increased catabolism of fever, but there is nothing in their report to indicate whether or not they gave sufficiently large doses to produce a distinct nephritis. Pearce and Eisenbrey[11] regard the earlier stages of the nephritis as physiologically tubular in character and the later stages as probably vascular.

Cat 23.—Weight 2,850 gm. Given 10 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 2d day. Well marked albuminuria on the 2d day. Found dead on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	41 mg.	25 mg.
Before injection (1 dy.)	42 mg.	28 mg.
24 hrs. after injection	50 mg.	33 mg.

Cat 24.—Weight 2,350 gm. Given 4.5 units of diphtheria toxin subcutaneously (about 2 units per kilo). Bled on the 3d and 5th days. No urine at the end of 24 hours; did not eat well after injection. Slight albuminuria at the end of 48 hours, increasing in amount until death. Found dead on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	38 mg.	22 mg.
Before injection (same dy.)	38 mg.	23 mg.
48 hrs. after injection	61 mg.	39 mg.
96 hrs. after injection	227 mg.	186 mg.

Cat 25.—Weight 2,950 gm. Given 9 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 3d and 4th days. Albuminuria appeared on the 3d day (urine lost on the 2d day), and was well marked until death. Found dead on the morning of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	41 mg.	24 mg.
Before injection (3 dys.)	41 mg.	26 mg.
48 hrs. after injection	50 mg.	36 mg.
72 hrs. after injection	200 mg.	158 mg.

Cat 26.—Weight 2,700 gm. Given 3 units of diphtheria toxin subcutaneously (about 1 unit per kilo). Bled on the 3d and 5th days. Albuminuria appeared on the 3d day and was moderate until death. Found dead on the afternoon of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	34 mg.	17 mg.
48 hrs. after injection	80 mg.	45 mg.
96 hrs. after injection	254 mg.	216 mg.

[11] Pearce, R. M., and Eisenbrey, A. B., *loc. cit.*

It will be seen that in the earlier stages, when the condition physiologically is tubular, there is only slight retention of nitrogen, but that later, when physiologically the condition is vascular the retention becomes extremely marked. This latter effect, however, probably has much to do with the increased catabolism of the intoxication, and, as Pearce and Eisenbrey point out, the vascular reactions are influenced at this stage by the profound and general effect of the toxin rendering accurate physiological conclusions impossible. From both histological and physiological studies there can be little doubt that there is severe glomerular change at this period of great retention, as well as distinct tubular change.

TARTARIC ACID NEPHRITIS.

That tartaric acid can produce a nephritis was first pointed out by Underhill,[12] confirmed and studied further by Underhill, Wells and Goldschmidt,[13] and also confirmed by Pearce and Ringer.[14]

The nephritis produced in the cat by the subcutaneous injection of tartaric acid (racemic, Merck) shows a most marked necrosis of the epithelium of the convoluted tubules, as described in dogs and rabbits by Underhill and Wells. There is cloudy swelling of the epithelium generally. There is much albuminous precipitate in the subcapsular space of the glomerulus, as described by Pearce and Ringer. In the later stages the epithelial change is only that of slight cloudy swelling of the epithelium of the proximal convoluted tubule and occasional glomerular tufts show slight swelling of the convoluted tubules. Two cases show well marked interstitial edema. Studies of the vascular reactions of this form of nephritis have not appeared.

[12] Underhill, F. P., The Influence of Sodium Tartrate upon the Elimination of Certain Urinary Constituents during Phlorhizin Diabetes, *Jour. Biol. Chem.*, 1912, xii, 115.

[13] Underhill, F. P., Wells, H. G., and Goldschmidt, S., Tartrate Nephritis, with Especial Reference to Some of the Conditions under Which It May Be Produced, *Jour. Exper. Med.*, 1913, xviii, 322; A Study of Renal Secretion during Tartrate Nephritis, *ibid.*, p. 347; A Note on the Fate of Tartrates in the Body, *ibid.*, p. 317.

[14] Pearce, R. M., and Ringer, A. I., A Study of Experimental Nephritis Caused by the Salts of Tartaric Acid, *Jour. Med. Research*, 1913, xxix, 57.

Cat 27.—Weight 1,650 gm. Given subcutaneously 0.75 gm. of tartaric acid in 6 c.c. of water, plus 4 c.c. of saturated sodium carbonate solution (about 0.45 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 4th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, but was absent on the 5th, 6th, and 7th days, reappeared on the 8th, 9th, and 10th days, and was absent on the 13th day. Killed with chloroform on the 14th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	27 mg.
Before injection (same dy.)	42 mg.	24 mg.
22 hrs. after injection	69 mg.	45 mg.
46 hrs. after injection	36 mg.	20 mg.
71 hrs. after injection	40 mg.	22 mg.
118 hrs. after injection	57 mg.	31 mg.
142 hrs. after injection	54 mg.	30 mg.
11 dys. after injection	50 mg.	30 mg.
13 dys. after injection	52 mg.	31 mg.

Cat 28.—Weight 2,150 gm. Given subcutaneously 1 gm. of tartaric acid in 5 c.c. of water, plus 7 c.c. of saturated sodium carbonate solution (about 0.5 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and continued on the 3d day. Found dead in cage on the morning of the 4th day probably as the result of heart puncture.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	46 mg.	27 mg.
24 hrs. after injection	154 mg.	116 mg.
45 hrs. after injection	222 mg.	181 mg.

Cat 29.—Weight 2,120 gm. Given subcutaneously 1.8 gm. of tartaric acid in 8.2 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 0.9 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 5th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, continued very small in amount, was barely perceptible on the 8th day, and became marked thereafter. Cat developed snuffles and was killed on the 13th day.

Time of bleeding	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	44 mg.	26 mg.
24 hrs. after injection	132 mg.	100 mg.
45 hrs. after injection	94 mg.	66 mg.
96 hrs. after injection	38 mg.	19 mg.
120 hrs. after injection	37 mg.	19 mg.
144 hrs. after injection	40 mg.	25 mg.
7 dys. after injection	40 mg.	23 mg.
10 dys. after injection	50 mg.	30 mg.
12 dys. after injection	50 mg.	29 mg.

Cat 30.—Weight 1,800 gm. Given subcutaneously 1.6 gm. of tartaric acid in 8 c.c. of water, plus 8 c.c. of saturated sodium carbonate solution (0.9 gm. of

tartaric acid per kilo). Bled on the 2d, 3d, 4th, and 6th days. Albuminuria appeared on the 2d day and continued until death. Found dead in cage on the morning of the 7th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	40 mg.	22 mg.
Before injection (same dy.)	41 mg.	24 mg.
24 hrs. after injection	166 mg.	120 mg.
48 hrs. after injection	229 mg.	190 mg.
73 hrs. after injection	156 mg.	119 mg.
129 hrs. after injection	130 mg.	100 mg.

Cat 31.—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid in 10 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 0.9 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and on the morning of the 4th day. Complete anuria at the end of 24 hours, and on the 2d and 3d days. Found dead in cage on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	40 mg.	26 mg.
22 hrs. after injection	133 mg.	100 mg.
48 hrs. after injection	210 mg.	160 mg.

Cat 31.—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid in 9 c.c. of water, plus 6 c.c. of saturated sodium carbonate solution (about 1 gm. of tartaric acid per kilo). Bled on the 2d, 4th, and 6th days. Albuminuria appeared on the 2d day and was marked until death. Developed snuffles and was killed with chloroform on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	47 mg.	30 mg.
24 hrs. after injection	50 mg.	32 mg.
72 hrs. after injection	277 mg.	218 mg.
120 hrs. after injection	382 mg.	312 mg.

It can be seen that the degree of nitrogen retention in these animals with tartaric acid nephritis is well marked, that it appears to bear a direct ratio to the dose of tartaric acid, and that it is most marked when the lesion histologically is almost purely tubular.

SUMMARY.

These studies, like all studies of the kidney, are difficult of interpretation because of the impossibility of producing a pure glomerular or pure tubular nephritis. Arsenic nephritis in its early stage is physiologically a vascular nephritis, but anatomically tubular, and shows in this stage almost no retention of nitrogen, although studies of urinary nitrogen indicate an increased catabolism. On the other hand, diphtheria nephritis in the early stages is anatomically a

nephritis with marked involvement of the glomerulus anatomically, but it is physiologically a tubular form, and in this early stage there is little or no nitrogen retention although studies of metabolism indicate that protein catabolism is increased by the administration of diphtheria toxin. In both cases, however, there appears to come a time when the excreting power of the kidney is exhausted and nitrogen accumulation occurs in the blood. This is much less marked in the arsenic nephritis, with less tubular change, than in diphtheria toxin nephritis with its marked tubular degeneration. This same late accumulation of blood nitrogen is seen in immune serum nephritis, where the tubular changes are persistent and relatively more severe than the glomerular change. It might well be said that this is no argument for the influence of the tubules in the excretion of waste nitrogen because of the general exhaustion of the organism as a whole and of the kidney in particular, and that no positive conclusions can be drawn is indicated by the opening sentence of this summary. Tartrate nephritis was at first considered as purely tubular; but the appearance of occasional glomerular change, as mentioned by Wells in one of his animals, and the fact that the presence of precipitated albumen in the subcapsular space, pointed out by Pearce and Ringer, probably indicates an increased permeability of the tuft capillaries, all lead to the conclusion that although the tubular change predominates, there is, possibly, slight alteration of the glomerulus. This form of nephritis shows the most marked retention of nitrogen, persisting even though the glomeruli show almost no change, tending to clear up with the progress of time and evidently also with the repair of the tubular change, and bearing a direct relation to the dose of tartaric acid and presumably with the degree of tubular change. It must be remembered, however, that the nephritis in the cases of greatest retention is a very severe form, and this again clouds the physiological interpretation of the results.

The study shows no reason for altering the conclusions of our earlier studies,[15] but from the interpretation accorded above it appears to throw more stress on tubular change as determining nitrogen retention. It confirms in addition the value of the methods used for studies of this type.

[15] Folin, O., Karsner, H. T., and Denis, W., *loc. cit.*

A NOTE ON NITROGEN RETENTION FOLLOWING REPEATED INJECTIONS OF NEPHROTOXIC AGENTS.*

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During the course of experiments previously reported[1] it was considered advisable to try to produce a subacute or chronic nephritis by the repeated injection of nephrotoxic agents and to observe the effects of the repeated injections on the amount of non-protein nitrogen in the blood. For this purpose uranium nitrate and diphtheria toxin were selected, and the technique detailed in the first study was followed. The experiments although not extensive are considered of sufficient interest to justify publication. An animal in which spontaneous chronic nephritis had occurred is included in the report because of the relation of this process to the experimental conditions studied and because of the close relation it bears to the work of Folin, Denis, and Seymour,[2] which shows that in human cases of chronic interstitial nephritis with hypertension it is possible to reduce the blood nitrogen practically to normal by the use of a low protein diet.

URANIUM NITRATE.

Cat 33.—Weight 2,670 gm. Given 0.00025 gm. of uranium nitrate daily (about 0.0001 gm. per kilo) for 5 consecutive days; then given double this dose for 12 consecutive days; then triple the primary dose for 5 consecutive days; and then

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[1] Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789. Karsner, H. T., and Denis, W., A Further Study of Nitrogen Retention in the Blood in Experimental Acute Nephritis, *idem*, 1914, xix, 259.

[2] Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press). Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

quadruple the primary dose for 7 consecutive days. The cat lived for 11 days more and was found dead in the cage on the morning of the 41st day. It was observed for 41 days and received a total of 0.018 gm. of uranium nitrate, 9 times the dose necessary to produce a marked nephritis in a 4,000 gm. cat. Albuminuria appeared to a slight degree on the 3d day, becoming marked 24 hours after doubling the dose, and continuing so until the dose was discontinued, when it became slight and remained so until death.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	43 mg.	28 mg.
3d dy. of experiment	43 mg.	27 mg.
5th dy.	40 mg.	25 mg.
Dose doubled		
7th dy.	40 mg.	25 mg.
9th dy.	40 mg.	26 mg.
11th dy.	40 mg.	25 mg.
14th dy.	41 mg.	25 mg.
16th dy.	41 mg.	22 mg.
18th dy.	43 mg.	28 mg.
Triple dose (18th dy.)		
23d dy.	53 mg.	36 mg.
Quadruple dose (23d dy.)		
25th dy.	54 mg.	39 mg.
28th dy.	91 mg.	67 mg.
30th dy.	126 mg.	113 mg.
Dose discontinued		
32d dy.	160 mg.	125 mg.
35th dy.	174 mg.	142 mg.
39th dy.	228 mg.	190 mg.

Found dead on the morning of the 41st day.

At autopsy there was present a slight acute valvulitis (mitral), probably the mechanical effect of the frequent heart punctures, a chronic adhesive pericarditis from the same cause, fat necrosis in the abdominal fat, but an apparently normal pancreas. The kidneys showed no change except that the glomeruli were white and prominent. Histologically the interstitial tissue shows between the tubules of the cortex numerous small areas of recent overgrowth, the nuclei being large, prominent, and vesicular. The glomeruli show a normal capsule and normal capsular epithelium. The subcapsular space is filled with a hyaline albuminous precipitate. The vascular tuft occupies about the normal space in the glomerulus but is almost devoid of blood; the endothelium shows extremely large frequent and vesicular nuclei, occasional mitotic figures, occasional lymphocytes and leucocytes and a well marked increase of collagenous intercellular material, apparently representing thickened capillary walls. Occasional proximal convoluted tubules are normal, but many more show hyaline casts and granular detritus within the lumen; in these tubules the epithelium does not project the normal distance above the basement, is narrowed, but shows no necrosis. The other tubules, particularly the loops of Henle and the distal convoluted tubules, show

an unusually large number of nuclei, in some cases there being two and even three nuclei in what appears to be a single cell body, although very few mitotic figures could be found. The blood vessels appear to be normal. The condition is interpreted as a subacute diffuse nephritis.

This animal had in the beginning functionally normal kidneys, which were also probably anatomically normal, for at the end of the experiment they showed neither grossly nor histologically the features of a spontaneous chronic nephritis. The repeated doses of uranium nitrate resulted in the appearance on the third day of albuminuria, which in this case can safely be interpreted as indicating functionally at least the presence of a nephritis. Not until after fifteen days had elapsed, however, was any demonstrable nitrogen retention observed and then after four daily administrations of triple the primary dose or 0.0003 of a gram of uranium nitrate per kilo, a single dose of 0.0005 of a gram being sufficient to produce a well marked nephritis in the cat.[3] This moderate retention continued until five days after the primary daily dose was quadrupled. For nine days after the drug was discontinued the nitrogen retention increased, a period during which the nephritis functionally became more marked, but in which the anatomical condition of the kidney, at least in so far as the epithelium was concerned, showed attempts at repair. It is hardly probable that the drug was retained in the body during the twelve days following the cessation of administration and hence the nitrogen retention and the final death of the animal must be looked upon as the effects of the artificially produced nephritis, even though the course of the experiment appeared to show nitrogen retention in a general way proportional to the dose of drug.

DIPHTHERIA TOXIN.

Three animals were given diphtheria toxin[4] in daily doses of 0.1, 0.25, and 0.5 of a unit per kilo respectively.

Cat 34.—Weight 3,050 gm. Given subcutaneously 0.3 of a unit of diphtheria toxin (about 0.1 of a unit per kilo) daily for 30 days with the exception of the 6th, 13th, and 26th days, making a total of 27 injections, or 8.1 units of toxin. Albuminuria appeared on the 7th day, after the administration of a total of 1.5 units of toxin, became marked on the 13th day, after the animal had received a

[3] Folin, Karsner, and Denis, *loc. cit.*, p. 790 (cat 3).

[4] The toxin was furnished us and titrated by Dr. Theobald Smith.

total of 3.3 units of toxin, and continued well marked until it was killed by chloroform.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	35 mg.	16 mg.
4th dy. of experiment	37 mg.	20 mg.
7th dy.	37 mg.	20 mg.
9th dy.	35 mg.	19 mg.
11th dy.	36 mg.	19 mg.
14th dy.	50 mg.	28 mg.
18th dy.	40 mg.	24 mg.
25th dy.	46 mg.	28 mg.
31st dy.	41 mg.	28 mg.

The autopsy showed nothing notable grossly other than the kidneys which were small, firm, and showed slight depressions in the outer surface along the lines of the larger veins; the cut surface showed almost no striations and the glomeruli were prominent and red. Histologically there is found no overgrowth of connective tissue about the surface veins or in any other part of the kidney. The glomeruli show well marked swelling of the capsular epithelium, and in many cases slight proliferation of the cells, but in no case are there found the crescentic figures of chronic capsular disease. The vascular tufts are filled with blood and although an occasional migrating leucocyte is found the tufts are practically normal. The subcapsular space contains a granular albuminous precipitate. The proximal convoluted tubules are normal except for occasional small areas of necrosis of the epithelium. The distal convoluted tubules show much formation of hyaline droplets in the epithelial cells. The tubules otherwise show finely granular albuminous material in the lumina and the larger collecting tubules show large numbers of hyaline casts. The blood vessels show nothing other than congestion. The condition is a subacute epithelial nephritis with a subacute capsular glomerulitis.

Cat 35.—Weight 2,850 gm. Given subcutaneously 0.7 of a unit of diphtheria toxin (about 0.25 of a unit per kilo) daily for 7 days. Bled on the 3d, 5th, and 8th days. Died on the 8th day as the result of heart puncture. Albuminuria appeared on the 4th day and continued in considerable quantity until death.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	41 mg.	24 mg.
Before injection (3 wks.)	41 mg.	23 mg.
In the interval the cat lost weight and was ill, but recovered about 1 wk. before continuation of the experiment.		
Before injection (same dy.)	37 mg.	23 mg.
48 hrs. after beginning of injection	37 mg.	23 mg.
96 hrs. after beginning of injection	37 mg.	18 mg.
168 hrs. after beginning of injection	180 mg.	144 mg.

The autopsy performed immediately after death showed nothing notable grossly. The kidneys show histologically a well marked acute change. Through-

out the organ there is a distinct perivascular infiltration of leucocytes, lymphocytes, and endothelial cells. The glomeruli show swelling, desquamation of the capsular epithelium, and occasionally pyknosis and karyorrhexis. The subcapsular space contains granular albuminous material and desquamated cells. The capillary tufts although containing blood show endothelial swelling, an occasional mitotic figure, karyorrhexis, and many leucocytes both within the capillaries and infiltrating into the tuft. The tubules show slight albuminous degeneration, particularly in the distal convoluted tubules, and the collecting tubules show a few hyaline casts. The blood vessels are normal, except that some of the smaller ones contain an excess of leucocytes. The condition is an acute diffuse nephritis with the most marked change in the glomerulus.

Cat 36.—Weight 2,270 gm. Given subcutaneously 2.5 units of diphtheria toxin (about 0.5 of a unit per kilo) daily for 5 days. Bled on the 3d and 5th days. Albuminuria on the 3d day, continuing until death. Found dead in cage on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	50 mg.	38 mg.
48 hrs after first injection	40 mg.	26 mg.
93 hrs. after first injection	222 mg.	182 mg.

The autopsy showed nothing notable grossly. Histologically the kidney differs from that of cat 35 only in that there is much less infiltration of leucocytes in the glomerular tuft and in the perivascular tissue of the organ.

In cats 35 and 36 the repetition of doses produced a greater degree of retention than the single dose would have produced, but the notable retention did not appear before the third day, as was also the case with the cats reported previously, which had been given a single larger dose of toxin. These two cats had a severe acute nephritis. Cat 34 with smaller doses developed albuminuria a full week before there was any nitrogen retention; the retention was then only slight and returned to normal in four days, even though there was a continued albuminuria, and the autopsy showed a nephritis of considerable duration. Anatomically this nephritis with its slight retention differed little in extent of involvement from that seen in the other two cats, although the functional powers were widely different. The length of time occupied in the experiment with cat 34 was more than sufficient for the development of immunity; hence the metabolic effects of the later doses would probably have little effect on protein catabolism and thus give less reason for calling on the kidney to excrete large amounts of nitrogenous waste. It would seem probable, therefore, that in acute diphtheria

nephritis the large final accumulation of nitrogen is dependent not only on the presence of the nephritis but to a large degree upon the increased protein catabolism.[5]

SPONTANEOUS NEPHRITIS.

Cat 37.—Weight 3,500 gm. On first examination and throughout the experiment the animal showed distinct albuminuria, but there were no casts in the urine. A diet of 80 gm. of chopped beef maintained a fairly constant weight. Since albuminuria was constant it will not be mentioned in the following notes.

Date.	Weight.	Diet.	Blood. Non-protein nitrogen.	Blood. Urea nitrogen.
Apr. 15	3,500 gm.	80 gm. meat	—	—
Apr. 17		80 gm. meat	69	35
Apr. 25		80 gm. meat	58	32
Apr. 26	3,400 gm.	160 gm. meat	—	—
Apr. 27		160 gm. meat	63	38
Apr. 29		160 gm. meat	66	50
May 1	3,600 gm.	160 gm. meat	67	50
May 3		50 gm. rice, 25 c.c. 15 per cent. cream	—	—
May 7		Same diet	54	40
May 14		(Diarrhea)	—	—
May 15		80 gm. meat; condition cleared up	—	—
May 21	3,200 gm.	80 gm. meat	72	56
May 23		80 gm. meat, 1 gm. sodium chloride	—	
May 31		80 gm. meat, 1 gm. sodium chloride	54	40
June 1		80 gm. meat	—	—
June 22	Experiment terminated. Animal killed with chloroform.			

At autopsy the heart was enlarged and had a distinctly thickened endocardium. The aorta was dilated in the ascending part. The liver showed a fatty deposit in the centers of the lobules. The kidneys were normal on the outer surface, but on the cut surface showed radial streaks of connective tissue in the cortex, especially prominent near the pyramid.

Histologically the gross findings in the heart and liver are confirmed. The kidney shows a slight overgrowth of connective tissue especially about the glomerular capsules and the blood vessels; associated in many places is moderate infiltration of lymphocytes. The glomerular capsules are slightly thickened and the capsular epithelium is normal. The subcapsular spaces contain a granular albuminous precipitate. The tufts are enlarged and show a distinct increase in

[5] Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

the number of nuclei; there is almost no blood in the tufts and the collagenous material is increased in amount, and often hyaline. The tubules are normal except that there is a moderate amount of albuminous precipitate in the lumina, the distal convoluted tubules show thinning and increased granulation of the epithelium, and occasional hyaline casts are found in the collecting tubules. The condition is a slight chronic diffuse nephritis with slightly greater quantitative involvement of the glomerular tuft than of other parts of the organ.

This animal as it first came to the laboratory presented in notable degree the features of a spontaneous nephritis. Under the favorable conditions of the laboratory the degree of nitrogen retention sank considerably during the course of one week, and then with several days of excessive protein diet rose slightly, especially in the urea fraction. Four days of extremely low protein diet resulted in a decrease of the total non-protein nitrogen, but still with a relatively high urea fraction. The diet had to be discontinued because a diarrhea developed. Quite unexpectedly, a week of normal diet resulted in an excessive accumulation of nitrogen, and then the addition of sodium chloride in small quantities was followed by a decrease in retained nitrogen. This study is suggestive in connection with the studies of Folin, Denis, and Seymour,[6] but it is not conclusive. The important facts demonstrated are that a mild chronic nephritis in the cat may be accompanied by a moderate retention of nitrogen, that the degree of retention is variable, and that moderately low and extremely low protein diets may be followed by a reduction in the non-protein nitrogen of the blood.

SUMMARY.

It is possible by means of repeated injections of uranium nitrate to produce in the cat a subacute or chronic nephritis which can progress to a fatal termination and show in its course increasing accumulation of non-protein nitrogen in the blood. This nephritis differs only slightly from the spontaneous chronic nephritis of the species. Repeated doses of diphtheria toxin produce a subacute form of nephritis with only temporary or slight retention, but nevertheless anatomically a well defined nephritis. The spontaneous nephritis studied resembles more closely that produced by uranium nitrate than that produced by diphtheria toxin both histologically and from the standpoint of blood analysis.

[6] Folin, Denis, and Seymour, *loc. cit.*

A NOTE ON THE SPECIFICITY OF CYTOTOXINS.*

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Since Metchnikoff and Landsteiner, working independently, demonstrated in the serum of rabbits treated with guinea pig spermatozoa the development of substances which immobilized and dissolved spermatozoa more or less completely, many attempts have been made to obtain cytotoxic sera specific for the different types of body cells.

It was early shown by von Dungern (1) that if rabbits were injected with the tracheal epithelium of cattle, the serum of the treated animals developed the power of causing the rapid disintegration of epithelial cells of that kind. He showed further that the serum was lytic for bovine erythrocytes, but that the antibodies possessed a greater affinity for tracheal epithelium. Metchnikoff (2) found that his spermatoxic serum was likewise hemolytic, but he maintained that the hemolytic power developed as the result of the injection of hemolytic receptors. Later investigators have obtained cytotoxic sera by injecting into animals of a foreign species the cells of practically every tissue of the body; leucocytes (Metchnikoff (3), Besredka (4)), liver (Delezenne (5), Deutsch (6)), kidney (Lindemann(7), Néfédieff (8), Bierry (9), Ascoli and Figari (10)), pancreas (Surmont (11)), adrenal (Bigart and Bernard (12)), thyroid (Gontscharukov (13), Mankovski (14)), heart muscle (Centanni (15)), ovary (Ceconi and Robecchi (16)), nervous tissue (Delezenne (17)), (Centanni (18)), syncytium (Ascoli (19), Liepmann (20)), etc.

Several methods have been employed for testing the immune sera as regards their specificity for the particular cells used as antigens. The methods of exposing cell emulsions to the action of the serum, as used by von Dungern in his trichotoxin studies, and by Flexner and Noguchi (21) in studies upon the action of snake venom on various organ cells, has not been found satisfactory for testing the specificity of cytotoxins. This may be due to the fact that one is dealing with the action of antibodies on dead and dying cells instead of on living functioning ones.

The technique which has been generally used consists in injecting the immune serum into the animal body, either subcutaneously, intraperitoneally, intravenously, or into arteries leading to particular organs. Functional disturbances and histological changes in various organs have served as criteria for determining the degree of specificity. More recently several other methods have been used, especially complement deviation and the epiphanin reaction. The results with the use of these methods are briefly summarized below.

Cytotoxic sera obtained by injecting the cells of an organ into an animal of a

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foreign species are relatively specific for the species from which the antigen was derived; that is, serious or fatal symptoms develop when animals of that species are treated with suitable quantities of the serum. Animals of other species are not, as a rule, so affected. These antibodies are spoken of as showing species specificity. Reports are conflicting as to whether or not they are to any degree specific for the particular organs or tissues used as antigens. That an absolute organ specificity does not exist is generally admitted, but many authors maintain, especially from a study of the lesions found in injected animals, that a relative specificity can be demonstrated. Lüdke and Schüller (22), for example, in a recent paper describe the production in dogs of a true nephritis with insignificant lesions in organs other than the kidney by the injection of the serum of rabbits immunized with dog kidney. Pearce (23), however, called attention to the fact, several years ago, that often the most striking lesions that one finds after the injection of a cytotoxic serum may be referable not to a direct toxic action on certain parenchymatous cells, but to the hemagglutinative and hemolytic properties of the serum, causing thrombosis, embolism, and hemorrhages, with secondary necroses in organs. Pearce argues further that since the cells of different organs evidently have certain receptor characteristics in common, it is hardly conceivable that specific somatogenic cytotoxins can be produced.

Beebe (24) has suggested that by the use of nucleoproteids instead of whole cells as antigens a closer approach to specificity can be reached. Wells (25), however, has recently pointed out that Beebe's suggestion is based on the false assumption that the nucleoproteids constitute the most important and most specific part of the cell.

Apart from its biological interest the problem is important on account of its bearing on the possibility of treating certain diseases, particularly cancer and exophthalmic goitre, with specific cytotoxic sera.

EXPERIMENTAL PART.

We have used a technique which appears to possess some advantages over previous methods; namely, the cultivation of tissues *in vitro*. By this method, as was suggested in a former communication (26), active living cells may be exposed to the action of immune sera, under conditions where the effects of such disturbing factors as agglutination, thrombosis, and hemorrhages can be eliminated.

Unfortunately the tissues that have been generally used by workers with other methods—liver, kidney, and other parenchymatous organs,—can not be cultivated satisfactorily in tissue cultures and therefore can not be utilized in experiments with the new technique. After a consideration of the tissues that might be used, rat sarcoma and rat embryo skin were chosen.

The next consideration was the choice of a foreign species which could be used for immunization against these tissues. It was necessary that two conditions be fulfilled: first, the plasma from normal animals of the species should be a suitable culture medium for rat tissues; and secondly, the animals should react well to the injections, that is, they should develop a strong cytotoxic serum. Guinea pigs fulfilled these two conditions. Two groups of guinea pigs were therefore injected, one with sarcoma, the other with rat embryo skin. Plasma from the two sets of animals was later used for tissue cultures for both sarcoma and skin.

Experiment 1.—The tissue for injection was prepared as follows: A sarcoma rat was bled to death and the tumor removed aseptically. The tumor tissue, which was extremely friable but not necrotic was cut up with scissors and washed with physiological salt solution to remove the excess of blood. The embryo skin was obtained from embryos near term. The skin was carefully dissected off, washed in salt solution, and finely divided. Very little blood was present.

Guinea pig 1 was inoculated subcutaneously through a Bashford needle with 0.15 gm. of rat sarcoma. Guinea pig 2 received a similar amount of embryo skin. Ten days later a second injection in each animal was made. Twelve days after the second injection each animal was bled according to the technique previously described (27) and plasma was obtained for tissue cultures. A normal guinea pig was also bled to obtain plasma for control preparations. Six series of preparations were made composed of rat sarcoma and rat embryo skin in plasma from each of the three guinea pigs. Each series was composed of fifteen preparations. They were incubated at 37° C. and examined daily. The controls of both sarcoma and skin in normal guinea pig plasma showed after twenty-four hours an active outwandering of cells among which mitotic figures were frequent. At the end of forty-eight hours growth was luxuriant in practically every preparation. The preparations containing immune plasma were, on the contrary, much less active, though the majority showed, especially on the third and fourth days, a fair outwandering of cells. A slight but definite difference was noted in the behavior of both kinds of tissue in the plasma from the two treated animals. For each tissue there was a more marked inhibition of growth in the plasma from guinea pig 1, injected with sarcoma, than in the plasma from the animal treated with embryo skin.

Experiment 2.—In order to secure a higher degree of immunization than was obtained in the first experiment, it was decided to use larger doses of tissue for injection. Guinea pig 3 received subcutaneously 0.65 gm. of rat sarcoma. Guinea pig 4 received a similar quantity of embryo skin, prepared as in experiment 1. Twelve days later plasma was obtained in the usual way from each animal, and from a third control normal guinea pig. Small pieces of sarcoma and skin were put up in the three kinds of plasma, making six sets, each composed of fifteen preparations. Examinations were made at the end of twelve hours and daily up to the sixth day of incubation.

The controls of both tissues grew well as usual, active outwandering of cells being noticed at the end of twelve hours. There was marked inhibition in the preparations of all four sets containing immune plasma. Many of the pieces of sarcoma and skin in plasma from both immune animals not only showed no growth but presented an amorphous granular appearance. Some of the preparations, however, showed after two days an outwandering of a small number of cells. As in experiment 1, a definite difference in the toxic power of the two immune plasmas was observed. In this instance, however, the more toxic plasma was that obtained from guinea pig 4, immunized with embryo skin. This plasma acted more strongly on both sarcoma and skin than the plasma from guinea pig 3, as shown by the more complete disintegration of the tissue fragments and the smaller number of cells which wandered out in the preparations that were positive.

Experiment 3.--The two guinea pigs used in experiment 2 received after bleeding second injections of 0.65 gm. of sarcoma and skin respectively. Ten days later they were again bled and their plasma was used for cultures of sarcoma and skin as before, with controls in normal guinea pig plasma. All the fragments of both sarcoma and skin in the immune plasma of both series showed rapid disintegration with no outwandering of cells. The controls showed excellent growths.

Experiment 4.—A guinea pig was injected intraperitoneally with 15 c.c. of defibrinated rat blood. Twenty-one days later plasma was obtained and used for culture preparations of sarcoma and skin. All preparations in normal guinea pig plasma showed active growth. Both tissues showed a feeble growth in a majority of the preparations in immune plasma. In the remaining negative preparations the disintegration of the tissue fragments was not so marked as in experiments 2 and 3.

DISCUSSION.

From the first experiment it is seen that relatively small doses of rat sarcoma or embryo skin are sufficient to induce in guinea pigs an antibody reaction such that the plasma of the treated animals becomes a poor medium for the growth of either sarcoma or skin cells. There was no evidence of even a relatively specific action on the part of the cytotoxins formed, the plasma from each animal exerting an equal inhibitory action on each kind of tissue. The plasma from the animal immunized with sarcoma proved to be slightly more active against each tissue than that obtained from the guinea pig immunized with embryo.

In the second experiment in which larger doses of tissue were used for immunization a more marked toxic action was demonstrable. Again no evidence of specificity was seen. As in experiment 1 plasma from one of the animals was found to be more active against both tissues. In this case, however, it proved to be that

obtained from the guinea pig immunized with embryo. It seems clear then that these differences in toxicity are to be referred to individual differences in guinea pigs in their reacting power and not to differences in the antigenic properties of the two tissues.

The third experiment shows that a still stronger cytotoxic serum may be obtained by a repetition of large doses of either tissue. Here again no evidence of specificity was found.

The fourth experiment shows that blood may be used to immunize against both sarcoma and skin, although the cytotoxic power of such a serum seems to be not so great as when either of the other tissues is used.

A second parallel set of experiments was carried out with two chick tissues, heart and intestine, for the production of cytotoxic sera in guinea pigs. The tissues were obtained from sixteen to twenty day chick embryos. These two tissues were selected because they exhibit characteristic types of growth in tissue cultures. From the heart a radial growth of connective tissue cells is regularly seen, while from pieces of intestine wide sheets of epithelial cells stretch out. Chick tissues grow fairly well in normal guinea pig plasma. In plasma from immunized guinea pigs the results were practically the same as those obtained with rat tissues; that is, no evidence of specificity was found. The experiments are therefore not given in detail.

SUMMARY.

1. The plasma of guinea pigs treated by injections of rat sarcoma exhibits a toxic action in tissue culture preparations on the cells of both rat sarcoma and rat embryo skin. Similarly, the plasma of guinea pigs immunized by injections of rat embryo skin is toxic for cells of both types.

2. Injection of rat blood immunizes against both sarcoma and embryo skin, although not so strongly as injections of the two tissues.

3. Guinea pigs receiving injections of either chick embryo heart or intestine develop cytotoxic substances for both of these tissues.

4. The preceding findings tend to show that cytotoxins formed after the injection of different body tissues into a foreign species are to no extent specific for the tissues injected.

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AN EXPERIMENTAL STUDY OF THE HISTOGENESIS OF THE MILIARY TUBERCLE IN VITALLY STAINED RABBITS.*

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Plates 36 to 39.

INTRODUCTION.

The inadequacy of the methods hitherto applied to the study of the histogenesis of the tubercle led us to produce tuberculosis experimentally in vitally stained rabbits. In rabbits injected with trypan blue the Kupffer cells stain deeply, while the blood cells are free from stain, and we thought that this might throw some light on the part played by the Kupffer cells in the formation of the miliary tubercle in the liver.

HISTORICAL.[1]

Opinion is still divided as to the origin and structure of the miliary tubercle. About fifty years have elapsed since Virchow first demonstrated the tubercle, named it, and showed that it was the distinctive product of tuberculosis. Although Virchow saw little in its morphology save round cells, it was not long before Langhans found the giant cell and noted its practically constant occurrence. Schueppel discriminated the three cell types (lymphocytes, epithelioid cells, and giant cells) which we recognize to-day as being usually present, if not of invariable occurrence, and of valuable diagnostic aid. The typical arrangement or succession of these three cell types has come to be a law (Ziegler).

Though we are now aware that they are by no means pathonomic for the tubercle, nevertheless the giant and epithelioid cells are peculiar structures, identical with none of the familiar fixed cells of the body, although descended from them. Their manner of descent and transformation have consequently claimed

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[1] The literature has been reviewed by Wechsberg, Dürck, Dürck and Oberdorfer, and others, and only the more important views will therefore be given.

much interest. After the discovery by Koch in 1884 of the etiological agent of tuberculosis, many investigators began to study the formation of the tubercle after the injection of pure cultures. One view identified with the name of Metchnikoff,[2] the other with that of Baumgarten, might be said to represent the views of the French and German schools respectively. Metchnikoff maintains that the tubercle is of leucocytic origin, and Baumgarten believes that the fixed tissue cells alone are responsible for its formation.

Metchnikoff's contention must be regarded as the natural outcome of his theory of the cells concerned everywhere in the defense of the body against bacterial disease. To the chief cells he has applied the term macrophage, meaning thereby a class of large mononuclear cells doubtless identified by others as wandering cells, which Metchnikoff and his followers considered to have arisen from the overgrowth of true lymphocytes. Stages in this transformation were described, so that there was no difficulty in imagining that with the increase in cell body, the deeply-staining lymphocytic nucleus became the large clear nucleus, poor in chromatin, of the epithelioid or giant cell. The phagocytic properties of the latter, however, were the chief cause of Metchnikoff's derivation of them from his macrophage, and he emphasized the almost invariable existence of bacilli in these cells. Unquestionably the convictions of Metchnikoff came more as a general result of his notions of the cells concerned in immunity than as the result of an exhaustive morphological research, but it is interesting to find that Koch himself was disposed to regard wandering cells as the chief elements in the tubercle, even though he imagined that their subsequent death might result in the participation of various fixed cells.

Baumgarten, and many investigators after him, have established the fact that the tubercle is not merely a heaping up of preformed elements, but that active proliferative processes are always concerned in its formation. The evidence for this was clear, for frequent mitoses are found, and inasmuch as these affect the chief cells of the immediate neighborhood, regardless of where the tubercle lies, Baumgarten and others have concluded that these cells alone are essential in tubercle formation, and that the most various tissue elements, including those as far apart as connective tissue and epithelium, could play this part, but that leucocytes could not. Indeed Baumgarten denied any but the latest and most trivial participation of leucocytes in the actual beginning of the tubercle, though subsequent investigators among his own countrymen could not, of course, confirm this. The preliminary outpouring of polymorphous cells and secondary lymphocytic infiltration which the French school had so clearly seen were undeniable, though Baumgarten's adherents denied a wider interpretation of this phenomenon as completely as the French school had denied the interpretation put on mitoses, the presence of which they also admitted. According to Baumgarten's theory, Kostenitsch and Wolkow describe the inception of a tubercle as follows: (1) the formation of a serofibrinous exudate, (2) migration of polynuclear leucocytes which rapidly disintegrate, (3) proliferation of fixed tissue cells to form epithelioid cells, (4) migration of mononuclear leucocytes which take a peripheral

[2] Metchnikoff's views are upheld by Yersin, Borrel, Leray, and Wallgren; Baumgarten's by Klebs, Kockel, Kostenitsch and Wolkow, Wechsberg, Miller, Watanabe, Oppenheimer, Straus, Morel, and others.

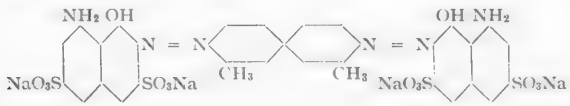
position around the epithelioid cells, and (5) secondary migration of polynuclear elements when the tubercle degenerates.

The question of the origin of the cells forming the tubercle depends on our ability to distinguish sharply cell types. Few reliable criteria have been advanced for such a discrimination, although the recent work of Wallgren and Oppenheimer should be mentioned. These investigators have taken advantage with us of the fact that the production of tubercles within the liver lobule permits us to work in a relatively simple territory, where besides the blood cells and endothelium of the hepatic sinuses, only the liver cells occur, all the connective tissue elements, for instance, being absent.

Oppenheimer, starting from the fact that silver particles are phagocytized largely by Kupffer cells, has produced hepatic tubercles after collargol injections. The tubercles which appeared after the transitory leucocytosis were composed of silver-containing epithelioid and giant cells, and Oppenheimer naturally derived these from the endothelium. It is unfortunate that the conclusiveness of his experiments is open to doubt on the grounds that normally some of the blood cells engulf the silver particles. Furthermore, Wallgren, also working with rabbit liver, has reached an opposite conclusion, and substantiates fully the lymphocytic origin of the cells. There appears to be sufficient reason, therefore, for a reinvestigation of the relatively simple liver tubercle with a more decisive method of study.

MATERIAL AND METHODS.

In our experiments rabbits were used exclusively. The animals were vitally stained by repeated intravenous injection of a freshly prepared aqueous solution of trypan blue. The dye is a benzidin of the following formula,



and has been used as a vital stain, by Bouffard, Goldmann, Schulemann, Evans, and others. The staining in the experiments to be recorded may be divided into two groups: the acute staining, where daily intravenous injections of twenty cubic centimeters of 1 per cent. solution of the dye were given during a few days, and which resulted chiefly in a pigmentation of the Kupffer cells; and the chronic staining where the injections were made at longer intervals and over a longer period of time. In these animals, besides the staining of the Kupffer cells, the blood of the liver capillaries contains many vitally stained macrophages. Both types of staining are adaptable to the study of the histogenesis of the tubercle, and the method of staining will be briefly given in each experiment.

Emulsions of bovine tubercle bacilli were made in the usual manner. The bacteria were carefully removed from the glycerin bouillon medium, dried on sterile filter paper, ground, and taken up in normal salt solution. This was then put into a shaking machine for two hours. The strength of the suspension was graded so that each cubic centimeter contained one milligram of dried tubercle bacilli.

The animals were anesthetized, a loop of bowel was exposed aseptically, and ten cubic centimeters of the suspension of tubercle bacilli were injected into a small radical of the mesenteric vein. The animals were killed at varying intervals, from one half hour to eleven days, and immediately injected through the ascending aorta, by pressure, with a 10 per cent. solution of formalin. Under these conditions the tissues are rapidly fixed throughout, the intra-acinar capillaries are dilated, and most of their blood content is washed out. The liver was then removed, cut into small strips a few millimeters in thickness, and replaced in formalin. Satisfactory results were obtained from frozen sections, and these were used throughout the study. With the above fixation, sections five micra in thickness were readily obtained. The sections were stained with aqueous cochineal, which contrasted well with the blue vital stain. Other sections were stained in Ziehl-Nielson carbol fuchsin for five minutes, decolorized in 10 per cent. hydrochloric acid alcohol, thoroughly washed in distilled water, and counterstained with Delafield's hematoxylin. Weigert's fibrin stain was used in some of the early stages.

THE RESULT OF THE INJECTION OF VITAL STAIN UPON THE LIVER OF NORMAL ANIMALS.

The literature on the histology of the normal rabbit's liver has been reviewed recently by Wallgren. Concerning the Kupffer cells, Wallgren quotes Schilling who considers them to be endothelial phagocytes and functional stages of liver endothelium. He believes that they arise from normal liver endothelium, by swelling and paling of their nuclei, and that probably every endothelial cell passes through this change from time to time.

In order to check Schilling's results, we killed an animal and in-

jected formalin through the aorta. The liver cells occasionally contain two nuclei, and a mitosis may occasionally be found. The nuclei of the endothelial cells of the capillaries are relatively wide apart, and two are found opposite each other only in rare cases. They are closely approximated to the columns of liver cells, and vary considerably in appearance. Many of them are flat, like ordinary endothelium, and there are transition forms to the larger ones with vesicular nuclei that bulge into the lumen of the capillary. The larger ones rarely contain an ingested polynuclear leucocyte or a granule of brown pigment.

EXPERIMENTAL PART.

VITALLY STAINED NORMAL ANIMALS.

Rabbit 1.—Mar. 14, 1912, 9 A. M. Injected with 20 c.c. of a 1 per cent. aqueous solution of dye in ear vein.

Mar. 15, 9 A. M. Killed. Fixed with formalin through aorta.

Liver.—The liver cells contain a few granules of trypan blue. The Kupffer cells are about normal in size and occurrence. Most of them contain a varying number of small blue granules. Endothelial cells with two nuclei or cells in mitosis are not found. Phagocytosis of polynuclear leucocytes or red blood cells is uncommon.

Blood.—The usual white blood cells, including lymphocytes and mononuclear and polynuclear leucocytes, occur and are unstained. Besides these large cells with clear non-granular protoplasm, vesicular nuclei and definite nucleoli occur. The nucleus may be round, oval, or bent. The nature of the cells is not always determinable. They appear to be similar to the large lymphocyte or polyblast emphasized by Wallgren.

Interstitial Tissue.—Normal; no vitally stained cells.

Rabbit 97 A.—Mar. 10 and 11. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of forty-eight hours.

Liver.—The cells contain a few small granules of vital stain. The Kupffer cells are more numerous, and many of them are increased in size. The majority contain blue granules of irregular sizes. These may be dense, or there may be only a few granules scattered near the nucleus, or even towards the tip of one of the protoplasmic processes of the cell. The dye is more abundant in the larger cells though the flat ones may contain a few granules. As the cell becomes larger, the nucleus becomes more vesicular, and rarely two nuclei may be found within one cell body. One mitotic figure was found. Phagocytosis occurs as usual.

Blood.—There are no vitally stained cells in the capillaries. The blood picture is similar to that of rabbit 1.

Interstitial Tissue.—An occasional clump of blue granules is found in the periportal tissue; but whether these are contained within the endothelium of the vessels or in the wandering cells cannot be determined.

Rabbit 200.—April 13, 14, 15, and 16. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of the fourth day.

Liver.—The cells contain definite, large, bright blue granules of dye, and may have two nuclei. The Kupffer cells are conspicuous. They are definitely increased in numbers and most of them are large and heavily laden with varying sized granules of dye. Some of the enlarged Kupffer cells are no longer firmly attached to the liver columns, and may be free in the lumen with their processes extending to the column of the liver cells. These cells may be cut in such a way as to appear round and free in the capillary. Not only are the Kupffer cells enlarged, but an occasional one with two or three nuclei and a rare mitotic figure may occur. In the latter the pigment granules are never found in the spindle. The nuclei of the large Kupffer cells may be irregular, and assume a polymorphous shape. The phagocytic property of the large Kupffer cells is demonstrated by the frequent finding of polymorphonuclear leucocytes or red blood cells within their bodies.

Blood.—The injection of formalin through the aorta was unsuccessful, and as a consequence, considerable blood is contained within the vessels. The larger veins contain normal unpigmented blood elements. The larger polyblast-like cells occasionally contain a few very fine granules of dye.

Interstitial Tissue.—There is an occasional pigmented cell, the nature of which could not be definitely determined.

Rabbit 80.—Six successive doses of 20 c.c. of 1 per cent. solution of dye were given on six successive days. The animal died shortly after the last injection, *i. e.*, early on the sixth day. The findings were similar to those in rabbit 200, except that two large pigmented giant cells were found in the liver.

Rabbit 49.—Nov. 23, 27, Dec. 6, 31, Jan. 5 and 13. 103 c.c. of 1 per cent. aqueous solution of trypan blue were given in approximately 20 c.c. doses. Jan. 19. Animal killed by a blow on the head, and 500 c.c. of 10 per cent. formalin injected through the aorta at a pressure of 80 mm. of mercury.

Liver.—The cells show a few granules of dye. Cells with two nuclei and occasional mitoses in the liver cells may be found. The Kupffer cells are conspicuous on account of their increased size and number, and their content of pigment granules. Kupffer cells with two or more nuclei are common and many large giant cells with innumerable nuclei are found. Frequently they occupy the position of the normal Kupffer cells, but sometimes they appear to be free. Mitosis is never demonstrable in them, but all transitions occur from those with two, three, and even more nuclei. The nuclei of the cells are large and twisted, so that they have a polymorphous appearance, and not infrequently the lobes may be connected by delicate threads of nuclear material. Phagocytosis is seen as usual.

Blood.—Besides the elements found in the previous cases, occasional large macrophages occur, often so densely pigmented that the nature of the cell is entirely obscured.

Interstitial Tissue.—The same as in the previous cases.

From the foregoing experiments it is evident that following successive intravenous injections of trypan blue the Kupffer cells become laden with the dye, enlarge, and increase in number, and finally form large free syncytial masses or giant cells containing one or

several nuclei. The endothelial cells which undergo this transformation and finally become large free macrophages are filled with increasing numbers of the vital blue granules in their cytoplasm. The blood cells, on the contrary, remain free from the dye and undergo no noticeable changes. It is important to note that whereas the dye thus stimulates the formation of free endothelial cells, it does so only after an interval of four or five days; within this interval only mild proliferative changes occur, resulting merely in the enlargement of Kupffer cells and their multiplication *in situ* in the vessel wall. We shall find in tuberculosis a far more rapidly changing picture.

EXPERIMENTAL TUBERCULOSIS IN UNSTAINED RABBITS.

Rabbit 101.—Killed forty-eight hours after inoculation with a suspension of 10 mg. of bovine tubercle bacilli in superior mesenteric vein. Dense cellular masses are scattered through the liver, either in the region of the periportal vessels or in the intra-acinar capillaries.

Liver.—The cells are coarsely granular and vacuolated, but otherwise present nothing of note. The Kupffer cells are more numerous than in the normal liver, and vary in size. Many of them are much larger, and as they increase in size the nuclei become more vesicular. The smaller, more slender Kupffer cells lie against the column of the liver cells like an endothelial membrane, while the larger ones seem to be less firmly attached and may be seen almost free in the lumina of the capillaries. Here their contour is easily made out. They are spindle- or winged-shaped, with long processes that often cross the bed of the capillary before they approximate themselves to the wall of the liver columns. When cut at different angles they often resemble polyblasts. They may contain polymorphonuclear leucocytes, which in many instances are well preserved and appear to be as healthy as those free in the capillaries. Around these ingested cells is a clear vacuolar space, which may cause an indentation in the nucleus where the latter comes in contact with the ingested cell. The ingested cell gradually undergoes disintegration. The chromatin may first disappear and leave a pale homogeneously staining protoplasm containing only a few chromatin specks, or the protoplasm may be digested or shrunken, leaving a small structureless mass of chromatin. Mitotic figures occur abundantly in the Kupffer cells, and Kupffer cells with two or three nuclei are found.

Blood.—The capillaries are dilated. Here and there in the smaller capillaries a few red blood cells or a polynuclear cell may be seen. Mononuclear cells also occur, some of which are small lymphocytes. By far the greater number, however, are irregular round or oval cells whose nuclei present no constant picture. They are apparently quite active, and while some have the chromatin arranged wheel-like, others are in division. Some of the cells are definitely Kupffer cells, since they are still attached by one or more processes to the columns of liver cells. Others are not in the capillaries, but occur as small nests of cells in

indentations between the liver cell and the endothelial lining. In one such area the Kupffer cell may be seen sending a process down between the liver cells. The larger clumps or plugs are in the periportal vessels, whose endothelium is swollen and partly desquamated. The lumen contains, besides these desquamated cells, one or more giant cells, polynuclear leucocytes, and mononuclear cells. One nodule, for instance, has a large, crescentic, multinucleated giant cell at one pole. The nuclei of this cell form a horseshoe, and several small beaded bacteria in the protoplasm occur. The remainder of the nodule is composed of numerous stellate spindle and oval cells with pale vesicular nuclei. Two are dividing. The vessel wall is not clearly distinguishable, and the cellular accumulation is present to a slight extent in the surrounding tissue. The giant cells, Kupffer cells, and spindle or oval cells contain the bacteria, which occur occasionally in polynuclear leucocytes also.

In some areas pictures occur which suggest the possibility that these giant cells may increase in size by fusion. Cells with nucleus and protoplasm similar to that of the giant cell are found in close apposition to each other.

Interstitial Tissue.—In the region of the periportal spaces the interstitial tissue is obscured on account of the frequent localization of the clumps in these areas, described above. Fibrin could not be demonstrated in the clumps.

Rabbit 84.—Killed seven days after inoculation.

Liver.—The section shows the liver tissue to be infiltrated with large nodules which tend to become confluent. The cells show nothing of note except an atrophy where the columns are pressed apart by the intravascular nodules. Kupffer cells are numerous. Many of them are large and multinucleated. Mitosis is frequent and was found in one cell with three nuclei. Cells may be seen dividing with the line of division perpendicular to the long axis of the vessel. Some of the Kupffer cells are more or less rounded and indistinguishable from the polyblasts in the capillaries except by their processes.

Blood.—The cell picture resembles that found in the previous experiment. Polyblast-like cells are abundant and many show mitosis. They are also found in nests beneath the endothelium.

Plugs.—The smaller plugs have a central giant cell surrounded by Kupffer cells and large round cells. The larger nodules show central caseation with a peripheral zone composed of the above cell types.

Bacteria are abundant and occur exclusively within the bodies of cells. The giant cells and particularly the Kupffer cells contain the organisms.

Summary.—The bacteria at the end of forty-eight hours are all intracellular, and frequent degenerative forms are encountered. The polynuclear leucocytes rarely contain tubercle bacilli, while the giant cells, Kupffer cells, and mononuclear cells contain them in the order named.

The Kupffer cells apparently are active. Frequent mitoses are found in them and Kupffer cells with two or more nuclei occur. These may form giant cells which may either occupy their normal

position or lie free in the lumen of the capillary. The giant cells do not show mitoses, but their nuclei are tortuous and polymorphous suggesting multiplication by budding. Elsewhere, within the center of tubercles pictures are occasionally encountered which suggest the possible formation of giant cells, or rather their increase in size by fusion, but these pictures are rare and not convincing.

Besides polynuclear leucocytes and definite lymphocytes in the capillaries, other large white cells occur which are difficult to classify. These cells are round or oval with homogeneously staining protoplasm and varying types of nuclei. They frequently show division. They are probably the cells spoken of as polyblasts by Wallgren. Some of them have streamers that extend to a neighboring Kupffer cell; some seem to be attached to the Kupffer cells, but the majority are free in the capillaries. Cells exactly like these may be found singly, or in nests lying in hollows of the liver cells below the endothelium. Individual cells of such a nest may be in mitosis. The Kupffer cell overlying the nest may also be in mitosis. The similarity between the cells of the nests and the large unclassified cells in the liver capillaries on the one hand, and the similarity and anatomical relation of both types to the dividing young Kupffer cells on the other hand are striking.

The nodules are of two types; those occupying the larger portal venules, and those of the intra-acinar capillaries. The first, even at the end of seven days, contain polynuclear leucocytes, though they are much less numerous than at the end of forty-eight hours. Besides these, large giant cells, stellate cells resembling Kupffer cells, and large mononuclear cells, compose the clumps. The vessel wall may or may not be intact in any one nodule. Outside the vessel similar cells may sometimes be found. Fibrin is not demonstrable.

The smaller intra-acinar nodules afford the more satisfactory study, consisting as they do of giant cells, which may or may not preserve their normal relation to the lining of the capillaries. Often the giant cell is pushed out and surrounded on all sides by cells similar to Kupffer cells, and by large mononuclear cells which may show mitosis, and occasionally by lymphocytes. These nodules completely fill the containing vessel, and in the later stages the centers may be caseous.

EXPERIMENTAL TUBERCULOSIS IN VITALLY STAINED RABBITS.

The difficulty of determining the origin of the cells of the young tubercle from ordinary stains is evident from the above description. It is impossible to say what the origin may be, beyond the fact that mononuclear cells are involved. Whether the endothelial or the mononuclear blood elements are concerned cannot be decided. An intermingling of the two cell types has taken place, and every transition from either type to the giant cell can be imagined. The vital stain, free from any possibility of inclusion of the blood cells, should furnish discriminating light here.

Rabbit 98.[3]—Killed twenty-four hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and one half hour after inoculation with tubercle bacilli.

Liver.—Aside from slight pigmentation, the cells show nothing abnormal.

The Kupffer cells are enlarged and relatively deeply stained. They correspond in size and intensity of vital staining to those of rabbit 97 A. Mitotic figures are found in much greater frequency than in the animals that received vital stain alone, and Kupffer cells containing two to three or four nuclei, though rare, may be found. The Kupffer cells show phagocytosis.

Blood.—Except for an apparent increase of polynuclear leucocytes there is no change in the blood picture.

Plugs.—Both in the larger interlobular veins and in the intra-acinar capillaries cell accumulations occur. The walls of the veins may be broken and their fibers stained vitally. Within the vein a bluish fibrillar substance occurs, in which many polynuclear leucocytes, a few red blood cells, and an occasional lymphocyte are enmeshed. In the center of these areas clumps of bacteria are frequently found. The surrounding polynuclear cells seldom contain bacteria, but the Kupffer cells frequently contain organisms even in distant areas. These cells may show mitosis, and a small group of cells may accumulate around them. Fibrin is not demonstrable.

Rabbit 100.—Killed thirty hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and six hours after inoculation with tubercle bacilli. Clumps of blue staining material scattered throughout the liver are readily seen with the naked eye.

Liver.—The cells contain granules of trypan blue. Many of the Kupffer cells are enlarged. Granules of vital stain are found in them as well as in the normal endothelial cells, but a number of cells are still unstained. Kupffer cells with several distinct nuclei occur occasionally. These may be so large that they almost occlude the lumen of the capillary. Mitoses occur in the Kupffer cells, but are not demonstrable in those with more than one nucleus. Phagocytosis is marked.

Blood.—The capillaries contain little blood. Here and there a few red blood cells occur. Polynuclears may also be seen, but they are not abundant. Some

[3] This animal was not injected with formalin after death on account of the risk of washing out the fresh capillary plugs in the liver.

of the mononuclear cells are definite lymphocytes, but others resemble polyblasts. None of the blood cells are pigmented with the vital stain.

Plugs.—Most of the plugs are small and lie in the intra-acinar capillaries which they may entirely occlude. They consist of large, swollen, pigmented Kupffer cells with varying numbers of large vesicular nuclei, a few polymorphonuclears, and an occasional mononuclear cell. The Kupffer cells are easily identified by their shape and pigment content. Some of them may be still attached to the vessel wall. They may contain bacteria, but this is difficult to demonstrate, since the bacteria are obscured by the vital stain. Here and there a mass of blue staining rods which resemble the bacteria occur. The larger plugs resemble those in the half hour stage.

Rabbit 97 B.—Mar. 10. 20 c.c. of trypan blue injected into ear vein.

Mar. 11. 20 c.c. of trypan blue injected in ear vein and inoculated with tubercle bacilli.

Mar. 12. Killed twenty-four hours after inoculation.

Scattered through the liver tissue are plugs of material conspicuous on account of their blue stain.

Liver.—The cells are normal in appearance and contain granules of trypan blue. The Kupffer cells are increased in size and number and contain many blue granules. The number of granules varies; some have only a few small ones, while others are so densely packed with them that the nucleus is obscured. Mitotic figures occur (figures 1, 2, and 3), and multinucleated cells are much more abundant than in the six hour stage. Phagocytosis is marked.

Blood.—The capillaries are dilated, but contain little blood. Polynuclears and red blood cells occur as usual. Mononuclears are more abundant. Most of them resemble polyblasts, but some are evidently Kupffer cells cut tangentially, and still have a process extending to the wall of the capillary. These have blue granules. Still others are found similar to them in every respect, but not attached to the vessel wall and without pigment granules. These cells are occasionally found lying in indentations of the liver cells below the Kupffer cells. With the exception of one polymorphonuclear leucocyte containing a few pigment granules, the cells of the circulating blood are normal.

Plugs.—These stand out sharply on account of the concentration of vital stain in them. Some of them consist of a single giant cell which is sometimes still attached to the vessel wall, and surrounded by only a few stellate Kupffer cells well laden with pigment granules. The larger plugs show a center of bluish material, in which polynuclear leucocytes and mononuclear cells are found. Surrounding these one or more pigmented Kupffer cells, or mononuclear, polyblast-like cells occur and form the remaining constituents of the plugs.

Bacteria.—A few bacteria are found in the Kupffer cells and in the giant cells. They are, however, made out with difficulty, as they are either stained or overshadowed by the trypan blue.

Rabbit 92.—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue. Inoculated with tubercle bacilli two hours later.

Mar. 3. Intravenous injection of 21 c.c. of trypan blue. Animal killed thirty-six hours after inoculation.

Liver.—The section is studded with many masses of vitally stained cells,

usually near the periportal spaces. The liver cells are coarsely granular and vacuolated. They contain a few small granules of trypan blue. The Kupffer cells are almost all enlarged and appear more numerous. The greater number of them are stained as usual.

A considerable number of cells with two, three, or more nuclei are found. These may still occupy their positions on the capillary lining. Giant cell formation is particularly marked (figures 5, 6, and 7). Mitoses are found in unusual numbers in Kupffer cells with one nucleus, but not in multinucleated cells.

Blood.—The capillaries contain red blood cells, and polynuclear leucocytes, but the mononuclear cells predominate. Some of them are lymphocytes, but the majority are similar to polyblasts. The latter are not infrequently seen in mitosis.

Plugs.—More numerous than in rabbit 97 B. In the larger ones, the polynuclear leucocytes have to a great extent disappeared. Deeply pigmented giant cells still partially attached to the vessel wall, stellate pigmented and non-pigmented Kupffer cells, and polyblast-like cells occur as usual. The polyblast-like cells in some places seem to push the giant cell away from its attachment to the vessel wall and infiltrate the surrounding tissue to a slight extent. Mitoses occur in these polyblast-like cells and are often demonstrable in the Kupffer cells in the vicinity of the larger plugs. The smaller intra-acinar plugs present the usual picture. They invariably contain a large pigmented multinucleated giant Kupffer cell (figure 8), and in some instances are surrounded only by irregularly pigmented Kupffer cells. The Kupffer cells vary in the amount of their pigment. In other instances polyblast-like cells are associated with the Kupffer cells at the periphery of the giant cells. These cells are not pigmented but sometimes cells almost identical in type contain a few granules. The smaller plugs may entirely occlude the capillary, and in some instances, where the giant cell is still attached in its original position, the lumen of the vessel may be entirely obstructed by it alone.

Bacilli.—Acid-fast involution forms occur rarely in the giant and Kupffer cells, but they are demonstrated with difficulty.

Rabbits 95 and 96.—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue, and 10 mg. of tubercle bacilli.

Mar. 3. Intravenous injection of 20 c.c. of trypan blue.

Mar. 4. Killed fifty-four hours after inoculation.

A detailed description of the cases will be omitted. Only the points will be emphasized which have not been noted in the previous experiments.

Liver.—The Kupffer cells show the usual pigmentation, mitosis, phagocytosis, and giant cell formation. One multinucleated Kupffer cell was found in which one of nuclei was in mitosis.

Blood.—The usual cellular elements are present. Polyblastic cells are the most conspicuous. They occasionally show mitosis, and some of them contain a few minute pigment granules. Cells similar to these, but more pigmented, occur singly or in nests in small pockets formed between the Kupffer cells and the liver cells. Individual cells in the nests may be undergoing mitosis. Their direct origin from Kupffer cells was not demonstrable, although large Kupffer cells in their vicinity, in some cases lying over the nest, were seen to be undergoing mitosis.

Plugs.—The larger plugs contain fewer polynuclear leucocytes; the smaller

ones are more cellular, otherwise they resemble those described in the previous experiment. In rare instances pigmented Kupffer cells may be so closely packed against a giant cell that the possibility is suggested of a giant cell increasing in size by fusion with Kupffer cells. In one tubercle occurring in a large periportal vein a tubular structure was found lined by swollen, pigmented Kupffer cells; at one end the cells dilated and filled the lumen, giving the appearance of a giant cell.

Rabbit 74.—Jan. 16. Intravenous injection of 14 c.c. of trypan blue.

Jan. 17. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 18. Intravenous injection of 20.5 c.c. of trypan blue and 10 mg. of tubercle bacilli.

Jan. 19. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 20. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 21. Killed seventy-two hours after inoculation with tubercle bacilli. The giant cells are particularly numerous and stained brilliantly with the vital stain. They are so often attached to the vessel wall that where this is not found it may be attributed to the plane of the section. One giant cell had three nuclei, one of which was undergoing mitosis. The polynuclear leucocytes in the circulating blood rarely contain a few blue granules. Kupffer cells are found free in the capillaries; they may retain their usual shape, or may be rounded with only a single process connecting them with the capillary lining. Such cells may be pigmented, and are hence distinguishable from the polyblastic cells.

Plugs.—In one of the plugs a non-pigmented giant cell was found. Pigmented and non-pigmented Kupffer cells, indistinguishable from polyblasts, may be found free in the capillaries. They may have several nuclei and the folding of the nucleus suggests an increase by budding. It is possible that these Kupffer cells are young, and the transition forms suggest that they may be the source of non-pigmented giant cells.

Experiments were carried on similar to those described in which the animals were killed at varying intervals up to eleven days. No new facts were brought out, however, and consequently a detailed description will not be given.

Rabbit 44.—A chronically stained animal had received the usual dose of trypan blue at regular intervals from Nov. 21 to Feb. 9, 160 c.c. of dye being injected during this period. On Feb. 15 the animal was inoculated with tubercle bacilli and on Feb. 19 a lobe of the liver was excised for study.

Liver.—The cells show the usual picture found in chronically stained animals. Large numbers of deeply stained macrophages occur throughout the liver capillaries. Definite tubercles were found in considerable numbers, usually located near the periportal areas. These are composed of central giant cells, and epithelioid and mononuclear cells. Occasional polynuclear leucocytes are found in the larger clumps. The point of chief interest, however, is that the tubercles contain practically no vital stain. The small amount of stain that is found in the tubercle appears as fine granules in the giant and epithelioid cells.

This case (rabbit 44) seems to us of interest from two points of view. First, the contention that preëxisting wandering cells form the tubercle is decisively disproven. There existed in this animal a great number of large free wandering cells electively stained so that their participation in the tubercle would have given us stained cells there. Secondly, the experiment demonstrates that the vital dye must be present in quantity in the body fluids so as to be accessible to

the new growth at the time of its formation. All our studies have shown the electivity of the tubercle for the vital stain, yet in rabbit 44, where no free stain was available, the tubercles were almost colorless. That failure to stain them vitally was not due to their leucocytic nature or to any refractility to the vital dye was shown by the experiments begun as was rabbit 44, but terminated by a vital dye of another color.[4] In these experiments red tubercles could be grown in blue livers.

Summary.—Within half an hour after the inoculation of tubercle bacilli in vitally stained animals cellular accumulations are found, particularly in the region of the periportal vessels and in the intralobular capillaries. The tubercle bacilli are found in clumps free in the centers of the larger cellular masses, but even in the early stages a large number of organisms are found in the bodies of the cells. The polynuclear leucocytes contain relatively few bacteria, but many of the Kupffer cells, even in areas remote from the cellular accumulation, contain organisms. In the later stages the clumps of free bacilli become less conspicuous, and organisms are demonstrable only in the bodies of Kupffer cells. The blue vital stain interferes somewhat with the demonstration of the organisms for it is very intense in the case of cells containing the bacilli. At the end of thirty minutes the plugs in the large vessels are partly composed of a homogeneous blue staining material in which are large numbers of polynuclear leucocytes. Occasional red blood cells and lymphocytes may be found here, but fibrin is not demonstrable. The vessel wall stains vitally, and this is probably the result of an injury brought about by the presence of the organism. The polynuclear leucocytes in the clumps gradually disappear and are replaced by cells which will be described below. At the end of half an hour mitotic figures are relatively frequent in the Kupffer cells, and this process continues through all the stages studied. As a result, Kupffer cells are found at the end of six hours with two or more nuclei, and at the end of twenty-four hours and later stages large brilliantly pigmented multinucleated giant cells lying in the position of the Kupffer cells form one of the most conspicuous elements in the section. The large Kupffer

[4] We employed dyes whose usefulness in this respect was discovered by Evans and Crowe (*Bull. Johns Hopkins Hosp.*, 1914 (in press)). Diamine fast scarlet 10 BF is a brilliant red which can be fixed in the tissues in formalin.

cells and giant cells[5] usually contain bacteria, and it is especially around these cells that the formation of the miliary tubercle may best be studied. The giant cell may lie partially or entirely free in the capillary connected only by a streamer to the lining cells. It is stained intensely vitally and, as a rule, it is surrounded by a varying number of cells which may all be stellate or wing-shaped Kupffer cells, containing more or less blue pigment. In this way a miliary tubercle may be found composed entirely of pigmented cells. Some of the Kupffer cells are not pigmented, in which case they are recognizable only by their peculiar form. This involves the separation of the non-pigmented cells from a third type of cell which closely resembles the polyblast. This cell is frequently found in the capillaries, and seems to occur in greater numbers after the six hour stage. Mitotic figures are found in them. They also occur singly, or in nests lying between the Kupffer cells and the liver columns. As a rule, they are not pigmented, but occasionally

[5] The tubercular giant cell noted by Rokitansky and Virchow, and emphasized by Langhans, Wagner, and Schueppel, has caused much discussion, particularly as to whether it is unicellular or multicellular in origin. Weigert, Straus, and Oppenheimer advocate the first theory; Kostenitsch and Wolkow, Kockel, Miller, and Watanabe consider that it results from a confluence of proliferated fixed tissue cells, and Metchnikoff, Borrel, and Wallgren think that it results from the fusion of white blood cells. Metchnikoff, however, has noted the formation of giant cells in the liver of *Spermophilus citillus* ("Ziesel") by a budding of the nucleus. Besides the true giant cells, Weigert, Arnold, Kochel, and others describe pseudogiant cells brought about by plugging of small vessels or bile ducts with exudate.

The nuclei of the large Kupffer cells are polymorphous and frequently several almost independent lobes may be found connected by slender strands of nuclear material. It seems probable, therefore, that the nuclei of the giant cells increase by direct division. Rarely pictures occur which suggest that the giant cells may increase in size by fusion with neighboring Kupffer cells. In the center of the larger tubercles deeply pigmented Kupffer cells closely approximated to the giant cell also suggest the possibility of this fusion. Consequently from the evidence that we have we should not deny the ability of more or less separate Kupffer cells to coalesce more perfectly to form the giant cells. But the endothelium of the hepatic capillaries is normally a common syncytial mass, and giant cell formation seems to us to be probably an intense unicellular response rather than an agmination phenomenon. A series of stages based on the unicellular hypothesis can be found in the growth of the young giant cell, although as a whole this question is of subsidiary interest compared with the manner of origin of the tissue.

a few fine granules of blue may be seen in the protoplasm. The cells occur abundantly in the larger tubercles.

Tuberculosis produced in chronically stained animals, in which no trypan blue was present in the circulation at the time of inoculation, resulted in the production of typical non-pigmented miliary tubercles. This is of interest because in chronically stained animals large numbers of deeply stained macrophages are always found in the circulation. These old macrophages are, however, seldom if ever concerned in the young tubercles that arise.

SUMMARY AND CONCLUSION.

In our study of the histogenesis of the miliary tubercle developing inside the liver lobule in animals that have been stained vitally while inoculated with bovine tuberculosis, the controls enable us to recognize the manner in which the vital stain affects the liver. There is therefore no possibility of confusing the effects due to the organism with the effects due to the dye. It is, however, of interest to note that the effects are closely related. The vital stain alone is able to produce gradually some of the same changes that occur with far greater rapidity in experimental tuberculosis. Although in a few hours the Kupffer cells of tuberculous animals begin to react to the disease, in the case of normal animals stained vitally they do not do this until after the third or fourth dose of successive daily injections. After many days, nevertheless, the vital stain alone produces enlargement, proliferation, and separation of Kupffer cells so that these are converted into large free phagocytes which may possess one or several nuclei. These are the gigantic macrophages of chronically stained animals. In all our experiments we have used only acutely stained animals, so that the effects of the dye itself are never sufficient to produce the changes. In fact there is no evidence that the dye accentuates the changes appreciably during the time involved in the experiment. The dye, however, shows us the type of the cells entering into the tuberculous granuloma, for when fed to the body fluids in abundance trypan blue finds its way into all cells capable of receiving it. The vital stain is, as it were, a physiological test for the cells. Whatever the fundamental nature

of the vital stain produced by trypan blue and the benzidine dyes may be, it is important that this reaction does not occur to any appreciable extent with mononuclear blood cells, and that it does occur emphatically in the case of the hepatic endothelium. By means of this vital test, then, the following phenomena occur when suspensions of tubercle bacilli are let into the portal blood stream. The organisms, swept on by the blood stream, finally lodge in the terminal branches of the portal vein, where they plug the vessels and continue to multiply. They injure the vessel wall and cause around them an exudative inflammatory process, and finally lead to the formation of tubercles situated not only in these areas but also within the liver lobule. The injury to the vessel wall is manifested in the early stages by the presence of vitally stained areas in its structure. The bacteria at the end of half an hour are found to be extracellular in clumps in the larger vessels, but already to some extent in the bodies of vitally stained Kupffer cells throughout the liver. Exudative inflammation manifests itself by the presence of a transitory accumulation of polynuclear leucocytes about the bacterial clumps, which may be seen as early as half an hour after the inoculation. They continue to be present in the larger cell clumps of the periportal areas for many days, but they are rapidly replaced by other cells, mononuclear in type, so that within a day the histological appearance of the portal plug has changed radically. The mononuclear cell thus entering most actively into the reaction is endothelial and not hematogenous in origin, the vital stain enabling us to make a clear distinction. This fact, evident in the portal plugs, is decisively shown in the case of tubercles developing within the liver lobule. Such tubercles probably result from the localization of individual organisms within the Kupffer cells, for the initial stages of such a probable cycle have been found by us. They consist of the occurrence of mitoses in certain Kupffer cells where the Ziehl-Nielson method shows a bacillus or several bacilli to have been phagocytized (figure 4). Rapid growth of the infected cell now takes place, and at thirty-six hours the multinucleated giant cell produced is largely separated from the other endothelium of the vessel wall. Many bacilli exist within the protoplasm of these cells (figure 5), which are especially distinguishable

by their intense reaction to the vital stain. They have received trypan blue to such an excess that low power views of liver sections at the thirty-six hour stage show these cells as deep blue spots (figure 8). The origin of the giant cell from the Kupffer cell is evident not only from the above sequence and from the elective stain, but also from the fact that even when fully formed, protoplasmic strands still join it to its mother tissue,—the normal endothelium of the vessel. The strands entangle other cells in their meshes, especially mononuclear blood cells, one of which, of the polyblastic type, has homogeneous protoplasm and is not infrequently encountered in mitosis. These cells are unquestionably of importance in the lesion of tuberculosis. We have seen them abundantly in the capillaries soon after the inoculation and they also occur singly or in nests between the Kupffer cells and liver columns. They are, as a rule, free from the vital dye. They continue to be concerned in the further growth of the tubercle and with the connective tissue cells make the structure of older tubercles relatively complex. On the other hand, little complexity occurs in the structure of the young intralobular masses. The miliary tubercle formed at the end of thirty-six hours is composed of a giant cell, surrounded by epithelioid cells and by blood cells of the above polyblastic type. The giant cell and its so called epithelioid cells are electively stained and are exclusively derived from the hepatic endothelium.

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EXPLANATION OF PLATES.

PLATE 36.

FIGS. 1, 2, and 3. Three stages in the mitosis of Kupffer cells from the liver of a rabbit twenty-four hours after inoculation with bovine tubercle bacilli. The large stellate endothelial cells are densely laden with the vital blue granules of trypan blue, 40 c.c. having been given intravenously in two doses. The section is counterstained with aqueous cochineal.

PLATE 37.

FIG. 4. Kupffer cell from the same rabbit as figures 1, 2, and 3. The section is stained by the Ziehl-Nielson method and shows the body of a Kupffer cell in mitosis containing three bacilli. The presence of these might be considered the immediate stimulus for growth of the cell.

FIG. 5. Young endothelial giant cell from the liver of a rabbit thirty-six hours after inoculation with bovine tubercle bacilli, stained as in figure 4 by the Ziehl-Nielson method. The cell is still connected to the vessel wall by protoplasmic processes; within it many bacilli and vital blue granules are found; four nuclei are included in the section; mitosis of any of the nuclei in the giant cell is rarely seen, so that direct division probably occurs.

PLATE 38.

FIGS. 6 and 7. Young endothelial giant cells from the liver of the same case as figure 5. The counterstain is aqueous cochineal. The enormous engorgement of these cells with the vital blue is readily seen. As yet no epithelioid cells surround the giant cells. The giant cell of figure 7 is already almost free, but that shown in figure 6 is still part of the endothelial wall, the nuclei of which had increased rapidly, so that a syncytial mass was formed.

PLATE 39.

FIG. 8. Low power view of a thirty-six hour old tubercle, showing the remarkable increase of the vital stain in the tubercle cells as compared with the remaining tissue. The tubercle is still almost purely the central giant cell, though several future epithelioid cells are noticeable.

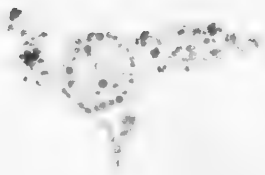

FIG. 1.

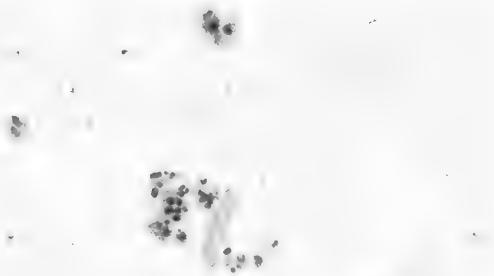

FIG. 2.

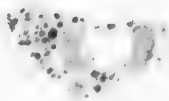

FIG. 3.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

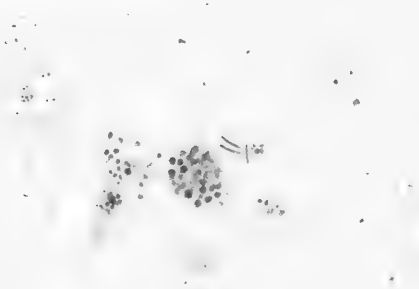

FIG. 4.

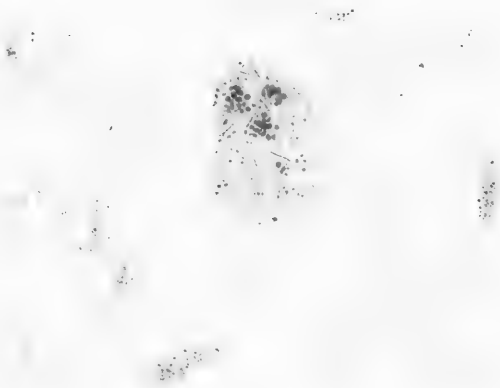

FIG. 5.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

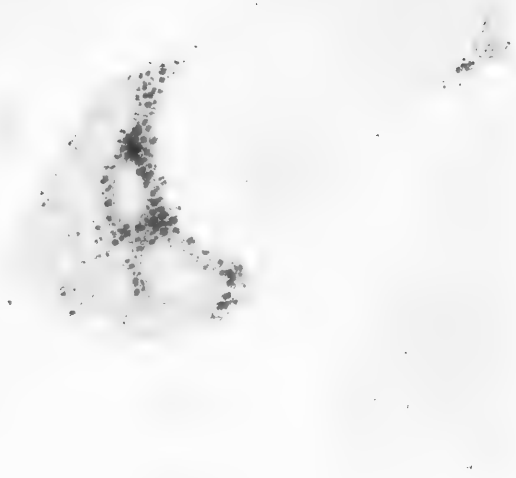

FIG. 6.

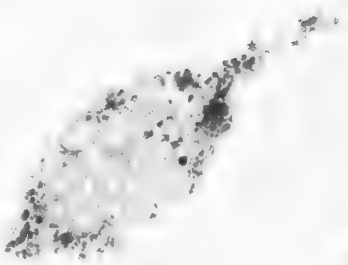

FIG. 7.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

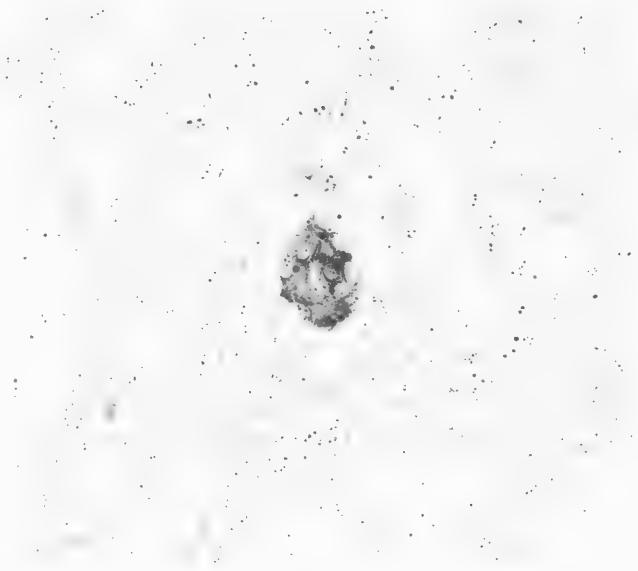

FIG. 8.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

THE INDOPHENOLOXYDASE CONTENT OF TISSUES FROM RABBITS INFECTED WITH PNEUMOCOCCUS.*

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The property of animal tissues to oxidize a mixture of naphthol and paraphenylendiamine into indophenol was first demonstrated by Römann and Spitzer.[1] Since then it has frequently been used as a test for oxydases, both in plants and animals. Recently Vernon[2] has devised a method for using this reaction quantitatively and has applied it to a number of problems, such as the quantitative distribution of the indophenoloxydase in various animal tissues, the relation of its action to lipoids, etc. According to Battelli and Stern,[3] indophenoloxydase belongs to the group of oxidizing agents which probably play an important part in the respiratory processes in the tissues. They call these oxidizing agents oxydones. The oxydones are not washed away from the tissues by water; alcohol and trypsin destroy them easily; the maximum activity occurs at 40° C.

It seemed of interest to study the behavior of the indophenoloxydase of animal tissues during pneumococcus infections. The problem has a bearing on the changes in the oxidation processes occurring in infectious diseases generally. In the present paper are recorded comparative figures showing the indophenoloxydase content of tissues from rabbits that died of general pneumococcus infection and of tissues from normal control animals.

METHOD.

The rabbits were given intravenously 0.2 to 0.5 of a cubic centi-

* Received for publication, January 15, 1914.

[1] Römann, F., and Spitzer, W., *Ber. d. deutsch. chem. Gesellsch.*, 1895, xxviii, 567.

[2] Vernon, H. M., *Jour. Physiol.*, 1911, xlii, 402; 1911–12, xliii, 96; 1912–13, xlv, 197; *Biochem. Ztschr.*, 1912, xlvii, 374.

[3] Battelli, F., and Stern, L., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 212.

meter of a freshly prepared virulent pneumococcus broth culture. Death followed usually after sixteen to twenty-two hours. The normal control animals were killed by a blow on the neck at the same time that the infected rabbits died, or within one to two hours later. The organs from both the infected and the normal animals were taken out, prepared, and kept under the same conditions.

The estimation of the indophenoloxydase content was made according to Vernon's[4] method. Five cubic centimeters of the reagent mixture, containing in 100 cubic centimeters a solution of M/1,500 α naphthol and paraphenylendiamine and M/620 sodium carbonate, are exposed to the action of 0.5 of a gram of the finely chopped tissue in Petri dishes, 8.8 centimeters in diameter, with frequently repeated stirring. The reagent solutions are mixed immediately before their addition to the minced tissue, and for each test a fresh pipette is used.

The time of exposure was one half hour for the renal cortex and the heart muscle, and one hour for the liver tissue. It was found that this period of time is sufficient to obtain definite information in regard to the activity of the indophenoloxydase present.

The indophenol formed is then extracted with ten cubic centimeters of 96 per cent. alcohol for one half hour, filtered, and its quantity calorimetrically estimated by diluting a certain number of cubic centimeters of it with 50 per cent. alcohol to the depth of color of the standard solution. Test-tubes of the same diameter and thickness must be used. The standard solution is prepared by diluting 1.4 cubic centimeters of the above mentioned reagent mixture to 200 cubic centimeters with 50 per cent. alcohol and keeping it three or four days, or until the maximum of color is reached. It contains 0.01 of a gram of indophenol in 100 cubic centimeters. The standard solution is renewed every week.

A large number of experiments have been made under various conditions. The more constant and reliable results have been obtained with liver, renal cortex, and heart muscle. They are recorded in the following tables, where other details of the experiments are also given.

[4] Vernon, H. M., *loc. cit.*

TABLE I.[5]

Pneumococcus Rabbit Died 22 Hours after the Intravenous Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed at the Same Time. Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4[6]	6.0	11.5	7.9	17.5	15.7	10 to 11 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	12.1	30 to 31 hrs. after death.
12.1	<3.0	12.7	6.0	—	—	3 days later.
Tissues autolyzed at 37° C.[7]						
12.1	4.0	10.3	7.2	17.0	15.7	10 to 11 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 to 31 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.

TABLE II.

Pneumococcus Rabbit Died 22 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 1 Hour Later. Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4	6.0	11.5	7.9	17.5	15.7	10 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	15.7	30 hrs. after death.
6.6	<3.0	11.5	5.4	—	—	15 dys. later.
Tissues autolyzed at 37° C.						
12.1	4.0	10.3	7.2	17.0	15.7	10 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.
6.0	<3.0	9.0	<3.0	—	—	15 dys. later. The tissues have been kept in the ice chest since the last experiment.

[5] The temperature at which the figures recorded in these tables were obtained varied between 22° and 28° C. Each normal control experiment was made at the same time and under the same conditions as the pathological.

[6] The figures that follow are not corrected for the spontaneous oxidation of the reagents into indophenol. On account of the short duration of the experiments (one half to one hour) the quantity formed is negligible.

[7] The autolysis at 37° C. was carried out in small Petri dishes 4 cm. in diameter, hermetically sealed with vaselin, in order to prevent the evaporation of the tissue water.

TABLE III.

Two Rabbits Have Been Inoculated Intravenously with Pneumococcus Broth Culture. Death Followed after 40 and 41 Hours Respectively. Normal Control Rabbit Was Killed 1 Hour Later.

Percentage of Indophenol Formed.

Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
15.7	15.0	15.0	15.7	12.0	22 and 24 hrs. after death. Tissues kept in ice chest.
9.1	<3.0	<3.0	4.2	<3.0	22 and 24 hrs. after death. Autolyzed at 37° C., kidney tissue for 7 hrs., heart muscle for 19 hrs.

TABLE IV.

Pneumococcus Rabbit Died 16 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 2 Hours Later.

Percentage of Indophenol Formed.

Liver.			Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.[8]	Pneumococcus rabbit.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
9.1	—	10.9	10.9	—	9.6	13.9	12.0	10 to 12 hrs. after death. Tissues kept in ice chest.
<3.0	<3.0	<3.0	9.1	9.1	4.2	10.2	7.2	10 to 12 hrs. after death. Tissues autolyzed at 37° C., liver and kidney for 3 hrs., heart muscle for 9 hrs.

TABLE V.

Normal Rabbit. All Tissues Were Kept at Room Temperature, 23° to 28° C.

Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
15.0	15.0	10.8	10.6	18.6	18.6	6 hrs. after death.
10.6	10.6	7.0	7.1	13.8	17.4	20 hrs. after death.
9.6	8.0	7.0	6.0	—	—	44 hrs. after death.
<3.0	<3.0	<3.0	<3 0	9.0	9.0	6 dys. after death.

[8] One drop of a freshly prepared pneumococcus broth culture was added to the normal tissue in all similar experiments.

TABLE VI.

Normal Rabbit.

Percentage of Indophenol Formed.

Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
14.1	—	27.0	—	6 hrs. after death. Kept in ice chest.
10.5	12.0	14.0	13.5	6 hrs. after death. Autolyzed at 37° C. for 3 hrs.

TABLE VII.

Normal Rabbit.

Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabb plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
		Tissues kept in ice chest				
17.7	—	10.3	—	—	—	8 hrs. after death.
18.0	—	10.3	—	22.7	—	14 hrs. later.
19.0	—	10.5	—	19.0	—	5 dys. later.
		Tissues autolyzed at 37° C.				
62.0	7.0	9.0	9.0	19.3	10.3	8 hrs. after death. Autolyzed at 37° C. for 2 hrs.
—	—	8.0	8.5	12.7	11.3	14 hrs. later. Autolyzed at 37° C. for 4 hrs.

SUMMARY AND CONCLUSIONS.

1. The action of indophenoloxydase is generally diminished in the tissues (liver, renal cortex, heart muscle) of rabbits that die of pneumococcus septicemia. The diminution is more frequent and marked in the liver and kidney than in the heart muscle.

2. The diminished activity of indophenoloxydase becomes more evident when the tissues undergo autolysis. At 37° C. this ferment is much more easily destroyed in the tissues of the infected animals than in those from normal control animals (tables I, II, III, and IV). Exceptions are rare and they occur chiefly with the heart muscle.

3. Normal rabbit tissues, inoculated *in vitro* with pneumococcus culture, do not lose their indophenoloxydase much more quickly than do the normal controls, when they are kept either at room temperature or at 37° C. (tables IV, V, VI, and VII).

4. It is therefore probable that the diminished activity of the indophenoloxydase of tissues from rabbits with pneumococcus septicemia is not due to the presence of the pneumococcus in these tissues, but that it is associated with a pathological change in the animal cell during life.

A COMPARATIVE STUDY OF THE RATE OF PROTEOLYSIS OF TISSUES OBTAINED FROM RABBITS INFECTED WITH PNEUMOCOCCI AND OF TISSUES FROM NORMAL RABBITS.*

By FLORENTIN MEDIGRECEANU, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

In the previous paper[1] it has been shown that the activity of indophenoloxydase undergoes a diminution in the parenchymatous tissues, especially the liver and kidney, of rabbits that died of a general infection with pneumococci. This ferment is more quickly destroyed during autolytic processes in the organs from rabbits infected with pneumococci than in those from normal control animals. The presence of the pneumococcus was excluded as being responsible for this difference. It was thought that a more rapid breaking down of the nitrogenous bodies, the proteins, might be associated with the increased lability of the indophenoloxydase in the tissues of the infected rabbits.

In order to obtain definite information on the rate of proteolysis in the tissues studied (liver and kidney), the following experiments have been made. Normal rabbits, weighing 2,000 to 2,500 grams, were given intravenously 0.2 to 0.5 of a cubic centimeter of pneumococcus broth culture. The experiments have been divided into two series, those dealing with non-perfused organs and those with perfused organs.

In the first series the organs were simply removed from the body after the animal's death. In the second series the animals were killed by a blow on the neck when the clinical symptoms were severe and when pneumococci began to appear in the circulation. Im-

* Received for publication, January 15, 1914.

[1] Medigreceanu, F., *Jour. Exper. Med.*, 1914, xix, 303.

mediately after the animals were killed they were perfused through the thoracic aorta with 0.8 per cent. saline solution. The perfusion lasted for about half an hour in each case, until the liquid running out became clear and the organs looked pale.

Normal control rabbits were killed in the same way and at the same time as the infected rabbits, or were killed when the infected animals died. All subsequent manipulations of normal and pathological material were carried out in the same manner.

The organs, after removal from the body, were thoroughly ground with half their weight of pure sterilized sand, diluted with their weight of 0.8 per cent. saline solution, and the mixture was centrifugalized; the precipitate was washed and again centrifugalized. The joined liquids were finally diluted with 0.8 per cent. saline solution so as to have a concentration in 100 cubic centimeters of about 0.3 to 0.5 of a gram of total nitrogen for the liver and of about 0.2 of a gram of total nitrogen for the kidney. Portions were taken for the determination of the total nitrogen, the total filterable nitrogen, and the amino nitrogen. The solutions, covered with a thick layer of toluol in narrow cylinders of the same diameter and thickness, were then submitted to autolysis at 37° C. During the course of autolysis, portions were withdrawn from time to time and the total filterable nitrogen and the amino nitrogen content were estimated. The total nitrogen was estimated according to the Kjeldahl-Gunning method, the filterable nitrogen by precipitating the coagulable proteins by heat and acetic acid, the amino nitrogen by Van Slyke's method.[2]

The filterable nitrogen was estimated to obtain information with regard to the disintegration of the complex protein molecules. The amino nitrogen should afford further knowledge concerning the extent of autolysis of the lower proteinic products, the peptones and the free amino acids. The results are shown in the protocols that follow.

[2] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 275.

SERIES A.

Non-Perfused Organs.

Liver.

Experiment I.

Total original nitrogen. gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[3]				Filterable nitrogen after autolysis.[4]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.3440	0.0888	25.8	0.0348	39.2	0.1370	39.8	0.0782	57.0	0.2020	58.7	0.1300	64.4	Infected rabbit died 18 hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed when infected animal died.
				Liver from pneumococcus rabbit									
0.3600	0.0938	26.0	0.0376	40.0	0.1790	49.7	0.1132	63.2	0.2450	68.1	0.1795	73.2	Ten hours elapsed between death of animals and beginning of autolysis.

Experiment II.

				Liver from normal rabbit									
0.4800	0.1800	37.5	0.0537	29.7	0.2330	48.5	0.1008	43.2	0.3300	68.7	0.210	63.6	Death of infected animal 33½ hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed at same time.
				Liver from pneumococcus rabbit									
0.416	0.1400	33.6	0.0514	36.7	0.2560	61.5	0.1521	59.4	0.2859	68.0	0.1714	60.0	16 hrs. elapsed between death of animals and beginning of autolysis.

[3] Autolysis at 37° C. lasted 19 hours in experiment I, and 20 hours in experiment II.

[4] Autolysis at 37° C. lasted 62 hours in experiment I, and 160 hours in experiment II.

Experiment III.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis. Total nitrogen. Gm. in 100 c.c.	Total nitrogen. Per cent. of total original nitrogen.	Amino nitrogen. Gm. in 100 c.c.	Amino nitrogen. Per cent. of total filterable nitrogen.	Filterable nitrogen after autolysis.[5] Total nitrogen. Gm. in 100 c.c.	Total nitrogen. Per cent. of total original nitrogen.	Amino nitrogen. Gm. in 100 c.c.	Amino nitrogen. Per cent. of total filterable nitrogen.	Filterable nitrogen after autolysis.[6] Total nitrogen. Gm. in 100 c.c.	Total nitrogen. Per cent. of total original nitrogen.	Amino nitrogen. Gm. in 100 c.c.	Amino nitrogen. Per cent. of total filterable nitrogen.	Remarks.
				Liver from normal rabbit									
0.4000	0.1160	29.0	0.0431	37.1	0.2224	55.6	0.1395	62.0	0.2688	67.1	0.1822	67.8	Infected rabbit died 40 hrs. after inoculation. Pneumococci present in blood. Normal rabbit was killed 2 hrs. later.
				Liver from pneumococcus rabbit									
0.2908	0.0936	32.2	0.0326	34.8	0.1760	60.5	0.1080	61.3	0.1832	63.0	0.1082	59.1	13 and 15 hrs. respectively elapsed between death of animals and beginning of autolysis.

Experiment IV.

				Liver from normal rabbit									
0.3930	0.1210	30.8	0.0413	34.1	0.1951	49.6	0.1067	54.6	0.3390	86.2	0.2028	59.9	Death of infected animal 27 hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed at same time.
				Liver from pneumococcus rabbit									
0.3600	0.0868	24.1	0.0311	35.8	0.2000	55.5	0.1012	51.1	0.2000	55.5	0.1196	59.8	8 hrs. elapsed between death of animals and beginning of autolysis.

[5] Autolysis at 37° C. lasted 33 hours in experiment III, and 23 hours in experiment IV.

[6] Autolysis at 37° C. lasted 77 hours in experiment III, and 160 hours in experiment IV.

Kidney.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[7]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
Kidneys from 2 normal rabbits									
0.2320	0.0890	38.3	0.0255	28.1	0.1550	66.8	0.0695	44.8	Infected rabbits died 27 and 33½ hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
Kidneys from 2 pneumococcus rabbits									
0.2550	0.1130	44.3	0.0315	27.7	0.1640	64.3	0.1111	67.7	8 and 16 hrs. respectively elapsed between death of animals and beginning of autolysis.

Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[7]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
Kidneys from 2 normal rabbits									
0.2180	0.0704	32.3	0.0228	32.4	0.1370	62.8	0.0934	68.1	Infected rabbits died 18 and 19 hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
Kidneys from 2 pneumococcus rabbits									
0.2590	0.0803	31.0	0.024	27.8	0.1530	59.1	0.1104	72.1	13 and 14 hrs. respectively elapsed between death of animals and beginning of autolysis.

[7] Autolysis at 37° C. lasted for 40 hours in both experiments.

SERIES B.

Perfused Organs.

Liver.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[8]				Filterable nitrogen after autolysis.[9]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.2115	0.067	31.7	0.0168	25.0	0.0721	34.0	0.0406	56.3	0.1128	53.3	0.0678	60.1	Infected rabbit was perfused 23 hrs. after inoculation.. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.3660	0.1020	27.8	0.0379	37.9	0.2230	61.0	0.1335	59.8	0.3210	87.8	0.2170	67.7	About 24 hrs. elapsed between death of animals and beginning of autolysis.

Experiment II.

				Liver from normal rabbit									
0.2566	0.0910	35.4	0.0249	27.3	0.1124	43.9	0.0579	51.5	0.1463	56.9	0.0898	61.3	Infected rabbit was perfused 23 hrs. after inoculation. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.3020	0.063	20.8	0.0249	39.5	0.1520	50.3	0.0935	61.5	0.2510	83.1	0.1576	62.8	About 24 hrs. elapsed between death of animals and beginning of autolysis.

[8] Autolysis at 37° C. lasted 20 hours in both experiments.

[9] Autolysis at 37° C. lasted 64 hours in both experiments.

Experiment III.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[10]				Filterable nitrogen after autolysis.[11]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.5126	0.1210	23.6	0.0450	37.1	0.3100	60.5	0.1925	57.1	0.3650	71.2	0.2915	79.8	Infected rabbit was perfused 24 hrs. after inoculation. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.2844	0.060	21.0	0.0271	45.2	0.1430	50.2	0.0885	61.8	0.2020	71.0	0.1292	63.9	About 4 hrs. elapsed between death of animals and beginning of autolysis.

[10] Autolysis at 37° C. lasted 19 hours.

[11] Autolysis at 37° C. lasted 62 hours.

Kidney.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[12]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Kidneys from 2 normal rabbits					
0.2120	0.080	37.7	0.0303	37.7	0.1530	72.1	0.0937	61.2	Infected rabbits were perfused 23 and 24 hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
				Kidneys from 2 pneumococcus rabbits					
0.1916	0.0726	37.9	0.0260	35.8	0.1327	69.2	0.0792	59.7	About 24 hrs. elapsed between death of animals and beginning of autolysis.

Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[12]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Kidneys from 2 normal rabbits					
0.2687	0.0978	36.3	0.0334	34.2	0.1920	71.4	0.1480	77.1	Infected rabbits were perfused 24 and 25 hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
				Kidneys from 2 pneumococcus rabbits					
0.1724	0.060	34.8	0.0189	31.5	0.1310	76.1	0.0930	71.2	About 7 hrs. elapsed between death of animals and beginning of autolysis.

[12] Autolysis lasted for 40 hours in both experiments.

SUMMARY AND CONCLUSIONS.

Liver.—The amount of filterable nitrogen in the samples before autolysis is too variable, both in the normal livers and in the livers from pneumococcus infections in the rabbit, for a constant difference to be obtained.

The amino nitrogen before autolysis is generally slightly increased in the livers of the infected animals, especially in the perfused specimens.

Most of the experiments show an increased disintegration of the livers of the infected animals during the first stages of proteolysis (twenty to thirty hours), both in the non-perfused and the perfused specimens. This increased disintegration is generally more evident for the complex proteins (filterable nitrogen) than for the lower products (amino nitrogen).

In the later stages the proteolysis of the blood-containing livers of the normal and the infected rabbits tends to an equalization, or the relations reverse, the proteolysis of the normal samples becoming more advanced. In the perfused specimens the differences in the rate of proteolysis are the same as in the earlier stages.

Kidney.—The rate of proteolysis shows no constant differences in the normal and infected tissues.